MOLECULAR BASIS OF THE KIDNEY FILTRATION BARRIER: ROLE OF THE NEPHRIN PROTEIN COMPLEX

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ACADEMIC DISSERTATION

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Back cover illustration: Electron micrograph of effaced murine podocytes after treatment with adriamycin.
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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to by their Roman numerals in the text.

I Ahola H, Heikkilä E, Åström E, Inagaki M, Izawa I, Pavenstädt H, Kerjaschki D and Holthöfer H. A novel protein, densin, expressed by glomerular podocytes. J Am Soc Nephrol 14(7):1731-7, 2003.

II Heikkilä E, Ristola M, Endlich K, Lehtonen S, Lassila M, Havana M, Endlich N and Holthöfer H. Densin and β-catenin form a complex and co-localize in cultured podocyte cell junctions. Mol Cell Biochem 305(1-2):9-18, 2007.

III Heikkilä E*, Juhila J*, Lassila M, Messing M, Perälä N, Lehtonen E, Lehtonen S, Verbeek S & Holthöfer H. β-catenin mediates adriamycin-induced albuminuria and podocyte injury in the adult mouse kidneys. Nephrol Dial Transplant (accepted for publication).

IV Heikkilä E, Ristola M, Havana M, Holthöfer H & Lehtonen S. Nephrin cooperates with Neph1/3 to induce cell adhesion associated with decreased tyrosine phosphorylation of nephrin (submitted)

* equal contribution

ABBREVIATIONS

AP-1	activating protein-1
aPKC	atypical protein kinase C
CAmKII	calcium/calmodulin-dependent protein kinase II
CASK	calmodulin-associated serin/threonin kinase
CD2AP	CD2-associated protein
CNF	congenital nephrotic syndrome of the Finnish type
DAG	diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
duf/kirre	dumfounded/kin of Irregular-chiasm-C
FSGS	focal and segmental glomerulosclerosis
GBM	glomerular basement membrane
GLEPP-1	glomerular epithelial protein 1
Grb2	growth factor receptor-bound protein 2
HSNL	hermaphrodite-specific neurons
HSPG	heparin sulphate proteoglycan
IP_3	inositol (1,4,5) trisphosphate
IrreC/rst	irregular-chiasm-C/roughest
JAM-1	junctional adhesion molecule-1
LAP	leucine-rich repeat and PDZ domain
LEF/TCF	lymphoid enhancer factor/T cell factor
MAGI-1/2	membrane-associated guanylate kinase inverted 1/ 2
MAGUIN-1	membrane-associated guanylate kinase-interacting protein 1
MCD	minimal change disease
MDCK	Madin-Darby canine kidney
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
MT 1-MMP	membrane type 1 matrix metalloproteinase
NMDA	N-methyl-D-aspartate
N-WASp	neural Wiskott–Aldrich syndrome protein
P-cadherin	placental cadherin
PA	puromycin aminonucleoside
PAN	puromycin aminonucleoside nephrosis
PDZ	PSD-95/disc large/ZO-1
PLC- γ1	phospholipase C-γ1
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol (4,5) bisphosphate
PIP_3	phosphatidylinositol (3,4,5) trisphosphate
PSD	post-synaptic density
RT-PCR	reverse transcriptase-polymerase chain reaction
SH2	Src homology 2
SD	slit diaphragm
Sns	stick and stones
TRCP6	canonical transient receptor potential 6
VE-cadherin	vascular endothelial cadherin
WT-1	Wilms tumor 1
ZO-1	zonula occludens 1

ABSTRACT

Proteinuria is a hallmark of kidney diseases and a consequence of defects in the glomerular filtration barrier. This sophisticated filter consists of glomerular endothelial cells, glomerular basement membrane and glomerular epithelial cells (podocytes), and together these layers form a size and charge selective barrier for plasma proteins. Podocyte foot processes form an intercellular contact, the slit diaphragm, which has been shown to be essential for preventing leakage of plasma macromolecules into urine. The slit diaphragm is a unique cell junction containing immunoglobulin superfamily proteins as well as components of adherens and tight junctions. The immunoglobulin superfamily member nephrin is crucial for the formation of the slit diaphragm since mutations in nephrin gene result in a severe nephrotic syndrome, congenital nephrotic syndrome of the Finnish type (CNF) that is characterized by massive proteinuria already *in utero* due to lack of the slit diaphragms. This thesis work has investigated the molecular mechanisms of how nephrin participates in the formation of the slit diaphragm.

Nephrin and its homologue Neph1 have large extracellular domains which bind to each other and this complex has been suggested to bridge opposite podocyte foot processes and thus form the slit diaphragm. The association of nephrin with adherens junction proteins has also been suggested to play a role in regulating slit diaphragm assembly. Tyrosine phosphorylation of the intracellular domain of nephrin creates binding sites for several signalling proteins which together with nephrin activate actin cytoskeletal organization, elevate intracellular Ca^{2+} levels or decrease apoptosis. However, very little is known about the role of nephrin in cell adhesion. In this thesis work we showed that nephrin is able to bind to another member of the Neph family, Neph3. We further showed that Neph1 and Neph3 were able to form cell-cell contacts alone, whereas nephrin needed to interact with Neph1 or Neph3 in trans-configuration in order to induce cell adhesion. Tyrosine phosphorylation of nephrin was decreased when it formed cell-cell contacts together with Neph1 or Neph3. We also identified densin as a novel component of the nephrin protein complex. Densin was shown to form a complex with adherens junction proteins, P-cadherin and β-catenin, and further it behaved in a similar fashion as adherens junction proteins in cell junctions indicating that it may take part in cell adhesion. These data extend the current understanding of the composition of the nephrin protein complex composed of immunoglobulin superfamily and adherens junction proteins. Furthermore, these results suggest that nephrin may cooperate with Neph1 and /or Neph3 in the formation of the slit diaphragm which associates with alterations of tyrosine phosphorylation status of nephrin.

Podocyte injury is a central event in the development of proteinuria and is characterized by loss of slit diaphragms, appearance of tight-junction-like structures, up/down-regulation of specific podocyte proteins and effacement of podocyte foot processess. In this thesis work nephrin associating proteins, densin and Neph3, were shown to be up-regulated in podocytes of CNF patients and nephrin deficient mice which share characteristics with podocytes observed in chronic kidney diseases. These

data indicate that densin and Neph3 may be involved in molecular pathways which lead to morphological alterations commonly seen in injured podocytes. β-catenin was shown to mediate adriamycin-induced podocyte injury in mice, since β-catenin deficient mice were protected from podocyte injury and β-catenin was up-regulated in podocytes after adriamycin treatment. These data suggest novel molecular mechanisms underlying podocyte injury.

REVIEW OF THE LITERATURE

1. The Anatomy and function of the kidney

Mammalian kidneys are bean-shaped paired organs that lie in the posterior abdominal wall. Kidneys are surrounded by fibrous capsules and they are divided into cortical and medullary regions. In mammals a single renal artery supplies each kidney and it divides into smaller arteries which ascend the cortex. There they enter the Bowmans capsule and end up into into a vascular tuft known as renal glomerulus, where blood is ultrafiltered. The ensuing primary urine is further concentrated and processed in various segments of the renal tubuli.

The single functional unit of the kidney consisting of glomerulus (Bowman's capsule and glomelular capillary tuft) and renal tubule is called a nephron. In humans each kidney contains about 0,8 to 1,2 million nephrons. The function of the glomerulus is to filtrate blood in a size and charge selective manner forming daily about 180 liters of primary urine which first enters the urinary space. From the urinary space the urine flows to the proximal tubule and is further transferred to different segments of Henle's loop, the distal tubulus, the connecting tubulus and to collecting ducts. This whole tubular system forms together with blood vessels the effective machinery responsible for concentration of urine, pH regulation as well as water and electrolyte homeostasis (Hallgrimsson *et al*., 2003).

2. The glomerular filtration barrier

The glomerular capillary wall forms the glomerular filtration barrier which is responsible for ultrafiltration of plasma so that macromolecules and blood cells are retained in the circulation. It is a highly specialized structure which consists of three layers together forming a size and charge selective filter. Sieving starts at the glomerular endothelial cell layer in which plasma is filtered through the endothelial fenestrations. Filtration continues in the glomerular basement membrane (GBM) which is composed of a central electron dense layer (lamina densa) surrounded by two electron lucent layers (lamina rara interna and externa). Highly differentiated glomerular epithelial cells, called podocytes, provide the final sieve for the filtrate through their specialized cell-cell contact, the slit diaphragm (SD) (See Figure 1).

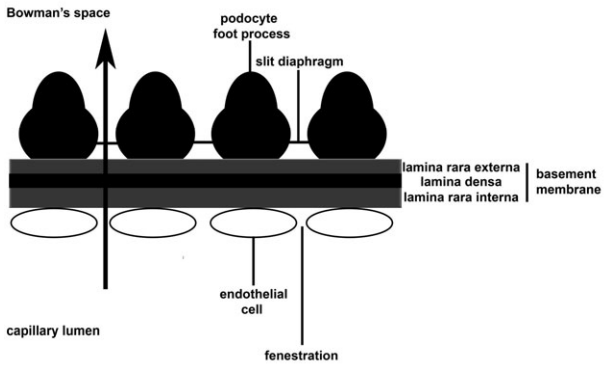

Figure 1: *Schematic presentation of the glomerular filtration barrier. An arrow indicates the direction of untrafiltration trough the filter.*

Glomerular endothelial cells

Vascular endothelium regulates blood flow and functions as a gatekeeper between blood and tissues by controlling the permeability for blood cells, proteins and other solutes (Minshall *et al*., 2006; Ley and Reutershan, 2006; Busse and Fleming, 2006). In the glomerulus, the function of endothelium differs from that in other parts of the body since it contributes to the formation of plasma ultrafiltrate of the blood and thus encounters high hydrostatic pressure. The plasma is filtered through trans-cellular holes (fenestrations) whose size (70-100 nm in humans) and number have an impact on the glomerular filtration (Satchell and Braet, 2009). The luminal side of the endothelial cells and fenestrations is covered by a layer of negatively charged membrane-bound macromolecules (proteoglycans, glycosaminoglycans, glycoproteins and glycolipids). The layer is called the glycocalyx and due to its negative charge, it is important for the permselectivity properties of the glomerulus (Pries *et al.,* 2000; Jeansson and Haraldsson, 2006). Endothelial cells are connected to each other via tight and adherens junctions and their integrity is also important for filtration (Bazzoni and Dejana, 2004; Kurihara *et al*., 1992; Sutton *et al*., 2003). Endothelial cells are also engaged in the formation of GBM by synthesizing its components, such as laminin (St John and Abrahamson, 2001) and type IV collagen (Abrahamson *et al.,* 2009).

Glomerular basement membrane

Basement membranes are extracellular matrices that surround endothelial and epithelial cells as well as individual adipocytes, muscle cells and nerve cells. In general, they provide support, divide tissues into compartments, and influence cell behaviour in multiple ways (Yurchenco *et al*., 1990). The GBM differs from other basement membranes in that it is exceptionally surrounded and maintained by two cell layers, the endothelial cells and podocytes. In electron microscopy the GBM can be separated into central electron dense layer, lamina densa, which is faced by electron-lucent layers, inner lamina rara interna and the outer lamina rara externa. The GBM is composed of glycoproteins including type IV collagen, laminins and entactin/nidogen as well as heparin sulphate proteoglycans (HSPG) such as agrin and perlecan. Type IV collagen and laminins form complexes which are connected by entactin/nidogen and this network

plays an essential role in size-selectively restricting the passage of the proteins through the glomerular filter (Abrahamson, 1987; Pihlajaniemi, 1996; Timpl and Brown, 1996). The importance of the laminin-type IV collagen complex for glomerular filtration is supported by the findings that mutations in genes encoding type IV collagen and laminin ß2 associate with congenital nephrotic syndromes, Alport syndrome (Alport 1927; Barker *et al.,* 1990) and Pierson syndrome (Pierson *et al.,* 1963; Zenker *et al.,* 2004), respectively. The level of HSPG, which binds to the collagen-laminin network, is decreased in patients with congenital nephrotic syndrome compared to healthy controls (Vernier *et al.,* 1983) emphazising the role of HSPG in developing charge-dependent permeability properties of the GBM.

Viscelar epithelial cells (podocytes)

Podocytes are highly differentiated epithelial cells with a complex cellular architecture. Their large cell body, bulging into the urinary space, gives rise to long primary processes. The processes extend toward the glomerular capillaries and divide into secondary foot processes which enwrap the capillaries by interdigitations. The basal membrane of podocyte foot process serves to anchor podocytes to the GBM via several adhesion molecules including $\alpha_3\beta_1$-integrin complex (Kerjaschki *et al*., 1989; Korhonen *et al*., 1990) and α–and β-dystroglycans (Raats *et al*., 2000). The apical membrane above the SD is highly negatively charged mainly due to a heavily sialylated glycoprotein podocalyxin (Kerjaschki *et al*., 1984). The foot processes from neighbouring podocytes are connected to each other via a special junction, the slit diaphragm (SD) (40 nm wide) (Rodewald and Karnovsky, 1974) (See Figure 2). SD is the only junction between the podocytes and it localizes between the apical and basal cell membrane domains of the foot processes. In electron microscopy the SD is seen as a filamentous strand which exhibits a zipper-like structure in which openings have dimension corresponding almost exactly to the size of albumin (Rodewald and Karnovsky, 1974). Therefore, SD has been suggested to play a crucial role in restricting passage of blood proteins. This hypothesis is supported by the finding that mutations in a gene encoding the SD protein nephrin associate with Congenital nephrotic syndrome of the Finnish type (CNF) (Kestila *et al.,* 1998). Nephrin deficiency in these patients leads to massive proteinuria associated with narrowing of the slits between podocyte foot processes and lack of the filamentous SD structure observed in electron microscopy (Patrakka *et al.,* 2000).

The unique shape of the foot processes is influenced by a highly organized actin cytoskeleton, whereas microtubules and intermediate filaments dominate in the cell body and primary processes. The actin cytoskeleton is important for the integrity of the SD and also for keeping podocytes attached to the GBM (Pavenstadt *et al*., 2003). Actin plays also a role when podocytes have to cope with mechanical stress from capillary wall tension as well as with shear stress which is a consequence of filtrate flow (Endlich and Endlich, 2006). Most of the cell organelles such as the nucleus, a well-developed Golgi system, endoplasmic reticulum, mitochondria and lysosomes are concentrated in the podocyte cell body, whereas primary and secondary foot processes contain only few organelles (Pavenstadt *et al*., 2003). The molecular composition of the GBM is important for podocytes to preserve their normal cell architecture. Therefore,

podocytes participate actively not only in adding components to mature GBM but also in turnover of the GBM by secreting matrix modifying enzymes (Abrahamson, 1985; McMillan *et al.,* 1996; Oneda *et al*., 2008).

The mesangium

Mesangium lies in the center of the glomerular tuft and provides structural support for the glomerular filter. It consists of mesangial cells which are surrounded by extracellular matrix (Zimmermann, 1933). Mesangial cells are irregular in shape and possess numerous processes which extend to the extracellular matrix and the GBM. They resemble vascular smooth muscle cells by having the ability to contract via their actin-myosin network and therefore they can regulate glomerular filtration via several vasoactive agents (Becker, 1972; Ausiello *et al*., 1980). They are also actively phagocytic and thus may contribute to the removal of glomerular debris (Mauer *et al*., 1972; Elema *et al*., 1976). Mesangial cells produce mesangial extracellular matrix which shares components with the GBM such as type IV collagen and laminins (Ishimura *et al.,* 1989). Mesangial cells bind to the extracellular matrix and regulate its composition and turnover (Veis, 1993).

3. Glomerular permeability

The glomerular permeability for macromolecules has been investigated by using tracers with varying charge, shape and size. Several tracer studies using dextran sulphate and positively charged ferritin have provided evidence that passage of negatively charged macromolecules through glomerular filter is more restricted than their neutral counterparts (Rennke *et al*., 1975; Chang *et al*., 1975; Bennett *et al.,* 1976; Guasch *et al*., 1993). Tracer studies have suggested that negatively charged lamina rara interna of the GBM and the glycogalyx lining the endothelial fenestrations form the primary barrier which restricts negatively charged molecules entering deeper in the GBM (Rennke *et al*., 1975). However, dextran sulphate tracer studies have also been criticized by showing that glomerular cells are able to take up these tracers (Tay *et al*., 1991) and plasma proteins, and that GBM can bind them (Vyas and Comper, 1994). Charge selectivity of glomerular filter has also been overruled by using fluorescently labelled albumin which has shown that tubular up-take is mainly responsible for restricting passage of proteins into urine and that this up-take system is impaired in proteinuric conditions (Russo *et al*., 2007). However, later on this was corrected by showing that the glomerular sieving coefficient for albumin is lower that it was reported in the fluorescently labelled albumin assays (Russo *et al*., 2007; Tanner, 2009). Furthermore, tubular up-take of albumin would be saturated if glomerular filtering would not exist (Lazzara and Deen, 2007). In addition, the results obtained from experimental animal models in which the charge of the glomerular wall is modified (Ciarimboli *et al.,* 1999) support the data obtained from tracer studies which, however, may need correction due to above mentioned limitations of tracer properties.

The size-selective properties of the glomerular filter has been investigated by using neutral ferritin (480 kDa) (Farquhar *et al.,* 1961) and dextrans with different molecular

sizes (32, 62 and 125 kDa) (Caulfield and Farquhar, 1974). The smallest dextran (32 kDa) was gradually lost from the blood and urinary space, whereas only minor amount of 62 kDa dextran (close to the size of albumin, 68 kDa) was detected in the urinary space and 125 kDa dextran was mostly retained in the circulation (Caulfield and Farquhar, 1974). Furthermore, several tracer studies using Ficoll (Blouch *et al.,* 1997; Ohlson *et al*., 2000) support the size-selectivity of the glomerular filter and based on the results, a two-pore model has been developed in which glomerular filter is suggested to contain large numbers of small pores (~ 37.5 Å) and low numbers of large pores (~120Å). According to this theory native albumin is normally passed through large pores but not through small pores. If the negative charge of the small pore is decreased, albumin is able to pass small pores which leads to increased amount of albumin in urine (Ohlson et al., 2001; Rippe and Haraldsson, 1994). The shape of the molecule has also shown to influence its permeability across the glomerular filter. Horseradish peroxidise, for example, which has similar globular molecular configuration as albumin, has been shown to be more restricted for transport across the filter than dextran that is a linear polymer (Rennke and Venkatachalam, 1979).

4. Why does the glomerular filter not get blocked?

There are several theories why circulating plasma macromolecules, which cannot pass the filtration barrier, do not block the glomerular filter. Mesangial cells (Farquhar and Palade, 1962) and podocytes (Farquhar *et al.,* 1961; Akilesh *et al.,* 2008) have been shown to clear the GBM by taking up proteins by phagocytosis, endocytosis or pinocytosis. The charge of the GBM has also been shown to prevent clogging (Kanwar and Rosenzweig, 1982). Smithies has provided an elegant gel permeation/diffusion theory in which the GBM is suggested to act as a size selective gel and SD provides resistance to fluid flow. According to this theory the local increase of albumin concentration inside the capillaries is lowered by red blood cells (having a diameter comparable to glomerular capillaries) which push away the excess albumin. The few macromolecules which enter the GBM pass it by diffusion rather than by liquid flow (Smithies *et al*., 2003). Based on the above, it may be that several anti-clogging mechanisms exist in the glomerular filter.

5. Congenital nephrotic syndrome of the Finnish type (CNF)

In 1956 Hallman and co-workers reported eight cases of infants in Finland who died of nephrotic syndrome, the eldest being 10 months. The symptoms included edema, increased plasma cholesterol, a low level of plasma albumin and massive proteinuria, which all are typical symptoms for a congenital nephrotic syndrome. The mothers delivered the babies up to six weeks prematurely and their placentas were exceptionally large, which indicates that the disease begins *in utero*. The kidneys of the affected infants were large and contained dilated tubules, mesangial hyperplasia and glomerulosclerosis (Hallman *et al.,* 1956).

Genetic basis of CNF

Ten years later Norio showed that the disease was inherited as an autosomal recessive trait and that it is more common in Finland than anywhere else, with the incidence of an estimated 1:8200 births (Norio, 1966) and, in consequence, it was termed as congenital nephrotic syndrome of the Finnish type (CNF). In 1998 the gene causing CNF was identified which was termed *NPHS1* and the gene product was named nephrin (Kestila *et al.,* 1998). Very soon after the finding of nephrin it was localized to SD in man and mouse by immunoelectron microscopy (Ruotsalainen *et al.,* 1999; Holzman *et al.,* 1999; Holthofer *et al*., 1999). Deletion of *NPHS1* gene in the mouse leads to morphological alterations resembling the findings in the CNF patients and therefore the mouse line provides a model to investigate pathogenesis underlying CNF. It is worth to note, however, that nephrin deficiency in mice is fatal earlier than in man resulting in death within 24 hours after birth (Putaala *et al.,* 2001; Rantanen *et al.,* 2002).

Pathological features of the CNF kidney

Macroscopical investigations have shown that the kidneys of CNF patients have a greater number of glomeruli and the glomeruli are about twice as big as normal (Tryggvason *et al.,* 1975; Tryggvason, 1978). The glomerular endothelial cells show formation of blebs, but the structure of the endothelium is mostly preserved (Kaukinen *et al.,* 2008). The mesangium is expanded due to mesangial cell proliferation and accumulation of extracellular matrix (Kaukinen *et al.,* 2010), but no alterations in the structure of the GBM is observed (Kaukinen *et al.,* 2008). Scanning electron microscopy has shown that podocyte cell bodies have balloon-like structures and instead of systemic inter-digitations of secondary processes, the capillaries are enwrapped by a flat cytoplasmic sheet. The number of podocyte slits was decreased up to 80 %, the width of the slits varied and about half of the junctions between the podocytes were only 5-10 nm wide thus resembling tight-junctions (Ruotsalainen *et al.,* 2000; Lahdenkari *et al.,* 2004). The fact that the CNF patients lack the filamentous image of SD in electron microscopy (Patrakka *et al.,* 2000) indicates that nephrin has a crucial function for the SD assembly and provides the first evidence that SD has a crucial role in the glomerular filtration barrier.

6. The slit diaphragm

The slit diaphragm arises from tight and adherens junctions during podocyte development

Podocyte development is divided into vesicle stage, S-shaped body, capillary loop and maturing stages (Reeves *et al*., 1978). Primitive podocytes appear first at the S-shape body stage during the glomerulogenesis and can be recognized by expression of podocyte-specific proteins including transcription factor Wilms tumor 1 (WT-1) (Mundlos *et al.,* 1993) and glomerular epithelial protein 1 (GLEPP-1) (Sharif *et al.,* 1998). At this stage podocytes form columnar epithelium in which the cells are connected with tight and adherens junctions that migrate down the lateral surface of the podocytes (Reeves *et al.,* 1978; Schnabel *et al.,* 1989). In the capillary loop stage podocytes lose their mitotic activity (Nagata *et al.,* 1993) and start to make interdigitations with early broad foot processes. Most of the processes are joined by

tight junctions, but junctions resembling SD can also be seen although they are wider (~90nm) and more apically located than in mature podocyte (Reeves *et al*., 1978). During maturing stage more capillaries are formed, foot processes are still differentiating and almost all of the tight junctions are replaced by the SDs (Reeves *et al.,* 1978) (See Figure 2).

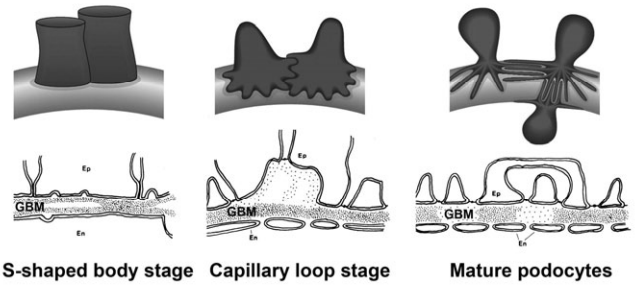

Figure 2: *Schematic presentation of podocyte differentiation. GBM, glomerular basement membrane; Ep, glomerular epithelial cells, podocytes; En, glomerular endothelial cells. Modified from Abrahamson 1987; Quaggin and Kreidberg 2008.*

Composition and morphological features of the SD

The tight junction protein Zonula occludens-1 (ZO-1) was the first protein which was localized to the mature SD and therefore the SD was first described as a derivative of the tight junction (Schnabel *et al.,* 1990). Later, other tight junction-associated proteins were detected in the SD including membrane-associated guanylate kinase inverted-1 (MAGI-1) (Hirabayashi *et al.,* 2005), MAGI-2 (Lehtonen *et al.,* 2005), calmodulin-associated serin/threonin kinase (CASK) (Lehtonen *et al*., 2004), junctional adhesion molecule-1 (JAM-1), occluding and cingulin (Fukasawa *et al.,* 2009). Tight junctions function in maintaining apico-basal polarity (Shin *et al.,* 2006) and they provide a cellular barrier for the transport of water, ions and proteins (Anderson, 2001). The SD polarizes podocytes, but in contrast to the tight junction, it contains a wider intercellular space (40 nm) and morphologically it resembles more adherens junctions (~20 nm). In fact, adherens junction protein P-cadherin has been localized to the SD and, therefore, the SD is suggested to be a modified adherens junction (Reiser *et al.,* 2000) (See Figure 3). The cytoplasmic area of the SD contains an electron dense region which shares similarities with cytoplasmic plaques of desmosomes. The intercellular width of desmosome (34 nm) is also close to that of SD (40nm) (Farquhar *et al*., 1961; Garrod and Chidgey, 2008), but in spite of that desmosomal components have not been detected in the mature SD (Garrod and Fleming, 1990). During differentiation podocytes seem to lose the typical epithelial cell characteristics and become highly specialized cells connected with a unique junction, that is, the SD.

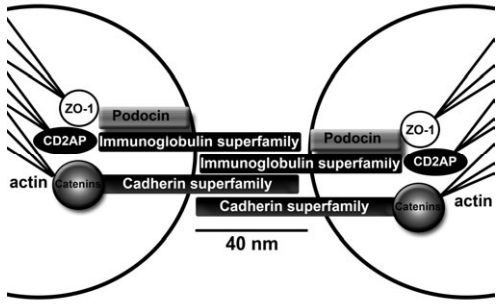

Figure 3: *The SD contains components of adherens and tight junctions as well as members of the immunoglobulin superfamily. ZO-1, zonula-occludens-1; CD2AP, CD2-associated protein.*

7. The role of nephrin in the SD

The crucial SD protein nephrin belongs to the immunoglubulin (Ig) superfamily of proteins which have been shown to act as adhesion molecules (Obrink, 1997; Irie et al., 2004). Nephrin has eight Ig-like domains, a fibronectin type III module and a short intracellular region containing nine potential tyrosine phosphorylation sites (Kestila *et al*., 1998). Based on the structure of nephrin and the finding, that without nephrin the SD cannot be formed, it was first speculated that nephrin would contribute to the adhesion and signalling pathways necessary for the SD assembly. More than ten years of research have provided evidence that nephrin, in fact, is a signalling molecule which participates together with other SD components in podocyte actin cytoskeleton organization as well as anti-apoptotic and Ca^{2+} signalling.

Nephrin is a signalling molecule

The first clue that nephrin may serve as a signalling protein was provided by showing that nephrin localizes in podocytes into lipid rafts (Simons *et al*., 2001), which are dynamic specialized plasma membrane assemblies enriched with signalling molecules (Rajendran and Simons, 2005). Nephrin has been shown to be tyrosine phoshorylated after antibody-induced clustering by Src family kinases (Lahdenpera *et al.,* 2003). Closer examination revealed that the Src family member Fyn, which has also been localized to the SD, was able to directly phosphorylate nephrin. In addition, Fyn deficient mice, which develop foot process effacement show decreased phosphorylation of nephrin supporting that the phosphorylation occurs also *in vivo* and it may have a role in podocyte injury (Verma *et al.,* 2003). Similarly, in puromycin aminonucleoside nephrosis (PAN) model in rat the tyrosine phosphorylation of nephrin is decreased (Zhu *et al*., 2008; Jones *et al.,* 2009). On the contrary, in experimental animal models of passive Heymann nephritis (rat) and protamine sulpfate nephrosis (mouse) which are both characterized by foot process alterations, the tyrosine phosphorylation of nephrin is increased (Li *et al.,* 2004; Verma *et al.,* 2006). It was also shown that tyrosine phosphorylation of nephrin was transiently seen during podocyte development in the capillary loop stage in mouse whereas the phosphorylation is not detected in mature murine podocytes (Verma *et al.,* 2006). Thus the balance between nephrin

phosphorylation and dephosphorylation seems important for the modulation of the SD structure.

Nephrin organizes podocyte actin cytoskeleton

Nephrin associates with actin (Yuan *et al.,* 2002) and also with SD and actin associating proteins, such as CD2-associated protein (CD2AP) (Lehtonen *et al.,* 2002; Palmen *et al.,* 2002), CASK (Lehtonen *et al.,* 2005; Biederer and Sudhof, 2001) and alpha-actinin-4 (Honda *et al.,* 1998; Lehtonen *et al.,* 2005) suggesting that it may organize podocyte actin cytoskeleton. Clustering of nephrin in the plane of plasma membrane has been shown to lead to tyrosine phosphorylation of nephrin by Fyn. The tyrosine phosphorylated nephrin is able to to bind to Nck and this complex induces actin polymerization which is likely mediated by Nck binding proteins and components of actin polymerization machinery, including neural Wiskott–Aldrich syndrome protein (N-WASp) and Arp2/3 (Verma *et al*., 2006; Jones *et al.,* 2006; Li *et al*., 2001) (Figure 4). Nck1/Nck2 deletion in mouse both *in utero* and in adult leads to podocyte foot process effacement resembling nephrin deficiency in mouse. This indicates that the nephrin-Nck interaction may be important also *in vivo.* Furthermore, the insufficient development of podocyte foot processes in Nck1/Nck2 deficient mice suggests that the Nck-nephrin induced actin polymerization may be important for foot process formation (Jones *et al.,* 2006; Jones *et al.,* 2009). The directed actin polymerization plays a central role also in the formation of cell-cell contacts by providing force to bring two plasma membranes in close vicinity (Vasioukhin *et al*., 2000). Therefore, the nephrin-Nck induced actin polymerization is also proposed to be involved in the formation of the SD (Verma *et al.,* 2006).

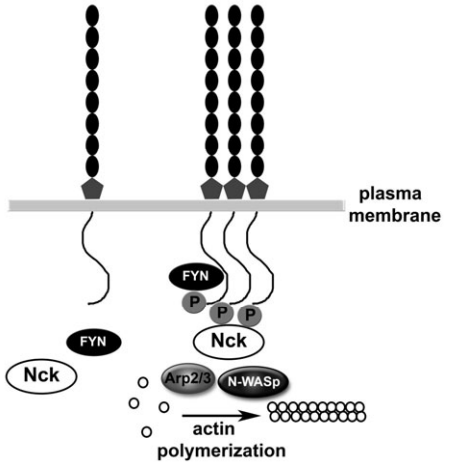

Figure 4: *Clustering of nephrin leads to its phophorylation by Fyn and consequent actin polymerization via Nck and actin organizing proteins Arp2/3 and N-WASp.*

Nephrin has also been shown to organize actin cytoskeleton by another signalling pathway. Nephrin phosphorylation by Fyn has been shown to create a binding site in nephrin for p85, which is a regulatory subunit of phosphoinositide 3-kinase (PI3K). The interaction results in the recruitment of catalytic p110 subunit of PI3K which leads to conversion of phosphatidylinositol (4,5) bisphosphate (PIP_2) into phosphatidylinositol-(3,4,5) trisphosphate (PIP_3) and activation of serine threonine kinase AKT. This signalling pathway was shown to decrease the formation of stress fibers in cultured rat podocytes (Zhu *et al.,* 2008). Nephrin-mediated PI3K signalling activates also Rac, a member of Rho family of small GTPases (Burridge *et al.,* 2004) and this pathway in turn was shown to increase formation of membrane ruffles in cultured rat podocytes (Zhu *et al.,* 2008).

The relevance of these *in vitro* formed actin structures in podocyte foot processes *in vivo* remains to be clarified. In cultured podocytes stress fibers have been shown to increase upon differentiation associated with the conversion of the cells with cobblestone characteristics into arborized morphology. Therefore, it has been speculated that these long actin filaments would be important for formation of podocyte foot processes (Mundel *et al.,* 1997). Plasma membrane ruffles (lamellipodia and filopodia) in turn are required for cell migration (Le Clainche and Carlier, 2008), which is suggested to play a role during foot process formation (Zhu *et al.,* 2008) and effacement (Reiser *et al.,* 2004).

Nephrin regulates Ca^{2+} signalling

Nephrin has also been shown to play a role in the modulation of cytosolic Ca^{2+} concentration via phospholipase C-γ1 (PLC-γ1). Clustering-induced phosphorylation of nephrin leads to binding and activation of PLC-γ1 which generates inositol (1,4,5) trisphosphate (IP_3) and consequently elevation of Ca^{2+} levels originating from endoplasmic reticulum (Harita *et al*., 2009) (Figure 5). Ca^{2+} spiking is known to induce various signalling cascades which lead to activation of cellular processes including differentiation, proliferation, and apoptosis (Berridge *et al.,* 2000). Since both nephrin and PLC-γ1 become phosphorylated in injured podocytes of protamine sulphate treated rats, it has been suggested that their activation may cause Ca^{2+} spiking which may induce morphological podocyte alterations seen in the rat model (Harita *et al*., 2009). Interestingly, mutations in a gene encoding PLC-ε1 associate with a nephrotic syndrome with diffuse mesangial sclerosis (Hinkes *et al.,* 2006). PLC-ε1 does not contain an SH2 domain which binds to tyrosine phosphorylated nephrin. However, since different subtypes of PLC have been shown to function together (Rebecchi and Pentyala, 2000), the hypothesis that PLC-ε1 would be in the same complex with the nephrin- PLC-γ1 complex and play a role in the regulation of podocyte morpholy together with nephrin and PLC-γ1 is an interesting possibility.

Nephrin interacts also with Transient Receptor Potential Cation Channel 6 (TRPC6) which is a calcium channel belonging to the canonical TRP subfamily that is activated by diacylglycerol (DAG) (Reiser *et al*., 2005; Clapham, 2003). TRPC6 has also been shown to be up-regulated in nephrin deficient mouse kidneys (Reiser *et al*., 2005). Even though the functional relevance of the interaction is not known, mutations in the gene encoding TRPC6 have been associated with focal and segmental glomerulosclerosis

(FSGS) (Winn *et al.,* 2005; Reiser *et al.,* 2005) making it an interesting binding partner for nephrin. *In vitro* investigations have shown that these mutations cause increased calcium influx into cells upon DAG activation indicating that Ca^{2+} spiking may trigger signals leading to podocyte injury in FSGS (Winn *et al.,* 2005). The TRPC6-mediated podocyte injury hypothesis is further supported by the data showing that the expression of TRPC6 is increased in podocytes in patients with membranous glomerulonephritis as well as in rat models of passive Heymann nephritis and PAN. Furthermore, PA-treated cultured podocytes show elevated Ca^{2+} influx upon DAG activation (Moller *et al.,* 2007). Since PLC- γ1 has been shown to bind and regulate TRPC3, which shares homology with TRCP6 (van Rossum *et al.,* 2005), and TRPC6 is activated through DAG and IP_3 produced by PLC-γ1 (Montell, 2005), it has been suggested that TRPC6 and PLC-γ1 may cooperatively mediate Ca^{2+} signalling in podocytes together with nephrin (Figure 5).

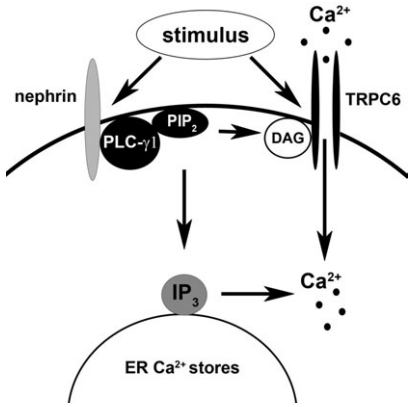

Figure 5: *Schematic presentation of the hypothesis how nephrin and TRPC6 may regulate Ca^{2+} signalling in podocytes. DAG, diacylglyserol; ER, endoplasmic reticulum; PIP_2, phosphatidylinositol (4,5) bisphosphate, PLC- γ1, phospholipase-C-γ1; IP_3, inositol (1,4,5) trisphosphate.*

Nephrin sends anti-apoptotic signals

Nephrin-dependent activation of PI3K-induced AKT signalling has also been shown to play a role in another cellular event, apoptosis. Nephrin-dependent AKT phosphorylation decreased apoptosis of cultured mouse podocytes via anti-apoptotic signalling protein Bad (Huber *et al.,* 2003). Similarly, nephrin binding partner CD2AP inhibited apoptosis in cultured podocytes. Furthermore, binding of CD2AP to nephrin facilitated AKT signalling suggesting that they may cooperatively inhibit apoptosis (Huber *et al.,* 2003). CD2AP deficient mouse kidneys show increased podocyte apoptosis compared to control mice (Schiffer *et al.,* 2004), whereas podocytes of neither nephrin deficient mice nor CNF patients are apoptotic (Kuusniemi *et al.,* 2006; Done *et al.,* 2008). The results indicate that nephrin loss alone does not induce apoptosis at least during development. However, this does not exclude the possibility that nephrin would play a role in apoptosis, for example, in acquired diseases in

adulthood. Furthermore, nephrin has also been shown to bind to dendrin, which is translocated to nucleus upon podocyte injury and induces apoptosis suggesting that nephrin may also regulate apoptosis via dendrin (Asanuma *et al.,* 2007).

Nephrin binds to evolutionarily conserved polarization complex

Nephrin has been shown to bind to Par3-Par6-atypical protein kinase-C (aPKC) complex (Hartleben *et al.,* 2008), which is an evolutionarily conserved cell polarization complex (Assemat *et al.,* 2008). The Par3-Par6-aPKCλ complex is essential for differentiation of immature spot-like (puncta) adherens junctions into belt-like and tight junctions, which is a crucial step for creating apico-basal polarity during epithelial cell polarization (Suzuki *et al.,* 2002). Similarly, in podocytes this complex is essential for polarization, because the podocyte-specific deletion of aPKCλ in mouse leads to mislocalization of the SDs which is associated with foot process effacement and proteinuria (Huber *et al*., 2009; Hirose *et al.,* 2009). During podocyte development Par3 and aPKCλ are present at the early S-shape body stage whereas nephrin appears at the late S-shape body stage and early capillary loop stage indicating that the Par3-Par6- aPKCλ complex could direct the localization of nephrin to the SD. Nephrin in turn may be important for stabilizing the nascent SD by organizing actin cytoskeleton (Huber *et al.,* 2009). It has also been shown that aPKCλ may play a role in localizing nephrin into lipid rafts (Hirose *et al.,* 2009).

8. Nephrin-like proteins

Establishing the Neph family

Donoviel *et al* identified nephrin-like protein termed Neph1 from the mouse gene trap database and consequently disrupted the gene by using the gene-trapping technology. Neph1 deficient mice developed podocyte foot process effacement, lack of SD and proteinuria, a phenotype that resembles nephrin deficiency (Donoviel *et al.,* 2001; Rantanen *et al.,* 2002; Putaala *et al.* 2001). The other Neph family members Neph2 and Neph3 (filtrin) were found by using Neph1 and nephrin sequences for database searches. Neph1-3 have similar structure composed of five extracellular immunoglobulin-like repeats, transmembrane domain and a short intracellular region (Sellin *et al.,* 2003; Ihalmo *et al.,* 2003). Furthermore, they all localize to the SD (Liu *et al.,* 2003; Barletta *et al.,* 2003; Gerke *et al.,* 2005; Ihalmo *et al.,* 2007), interact with SD protein podocin similarly as nephrin, and also bind to actin binding scaffolding protein ZO-1 (Sellin *et al.,* 2003; Huber *et al.,* 2003). In addition, Neph1 and Neph2 have been shown to form homodimers and heterodimers with nephrin (Liu *et al.,* 2003; Gerke *et al.,* 2003; Gerke *et al*., 2005; Barletta *et al.,* 2003). Since many immunoglobulin superfamily proteins play a role in the formation of the cell-cell contacts via homophilic and heterophilic interactions (Obrink, 1997; Irie *et al.,* 2004), it has been suggested that nephrin and Nephs would act in the same way in SD.

Neph1 is a signalling and actin organizing protein

Neph1 localizes to lipid rafts, the plasma membrane signalling centres, suggesting that it may act as a signalling protein similarly as nephrin (Barletta *et al.,* 2003). Indeed, Neph1 has been shown to activate transcription factor activating protein-1 (AP-1) (Sellin *et al.,* 2003), which mediates gene regulation responses leading to various cellular events (Hess *et al.,* 2004). Furthermore, Neph1 dependent AP-1 activation is augmented by the Tec family of tyrosine kinases and ZO-1 interaction (Sellin *et al.,* 2003; Huber *et al.,* 2003).

Neph1 is also tyrosine phosphorylated by Fyn which enables Neph1 to bind to growth factor receptor-bound protein 2 (Grb2). The activated complex has been shown to suppress Ras-Erk signalling cascades (Harita *et al.,* 2008; Lowenstein *et al.,* 1992) and induce actin polymerization (Garg *et al.,* 2007) presumably by activating N-WASp/Arp2/3 complex (Carlier *et al.,* 2000). It has also been shown that the activated Nephrin-Nck and Neph1-Grb2 complexes are able to cooperatively induce actin polymerization (Garg *et al.,* 2007). Furthermore, in a rat PAN model both nephrin and Neph1 show increased tyrosine phosphorylation associated with stronger interactions with their adaptor proteins Nck and Grb2, respectively. Therefore, the nephrin and Neph1 induced actin polymerization may play a role in disrupting the SD leading to podocyte foot process effacement in the PAN model (Garg *et al.,* 2007).

Neph2 is cleaved by a protease

The function and importance of Neph2 for the SD is largely unknown. Similarly as Neph1, it is known to activate AP-1 and it also contains a binding site for Grb2 suggesting that it initiates signalling cascades in podocytes (Sellin *et al.,* 2003). It has been shown that the extracellular domain of Neph2 can be cleaved by membrane type 1 matrix metalloproteinase (MT1-MMP). Furthermore, it has been shown that patients with membranous glomerulonephritis showed increased amounts of extracellular fragments of Neph2 in urine compared to healthy controls. In addition, the intracellular domain of Neph2 was also shown to be cleaved. However, the biological function of these Neph2 fragments is unknown (Gerke *et al*., 2005).

Neph3 is down-regulated in acquired proteinuric kidney diseases

The *NLG1* gene encoding Neph3 localizes next to *NPHS1* gene encoding nephrin in chromosome 19 (19q13.12) and they are transcribed to opposite directions. Therefore, it has been suggested that *NLG1* and *NPHS1* may share common regulatory elements for gene expression (Ihalmo *et al.,* 2003). The mRNA expression of nephrin and Neph3 are both down-regulated in the kidneys of diabetic nephropathy patients. In addition, their expression levels show positive correlation, which indicates that they may have common gene regulatory mechanism (Toyoda *et al.,* 2004; Ihalmo *et al.,* 2007). The mRNA expression of Neph3 is also down-regulated in other human proteinuric diseases including FSGS, minimal change disease (MCD), hypertensive nephropathy and membranous glomerulonephropathy indicating that Neph3 loss may be a general phenomenon in podocyte injury (Ihalmo *et al.,* 2007). Since Sp1 and NF- κB transcription factors have been shown to regulate Neph3 gene expression under basal conditions (Ristola *et al.,* 2009), they may also play a role in regulating Neph3

expression under pathological conditions, which, however, remains to be clarified. As in the case of Neph2, the importance of Neph3 for the formation of the SD is not known.

9. *The role of nephrin and Nephs orthologues in Drosophila and C. elegans*

Genes encoding nephrin and Nephs are conserved throughout evolution. Even though *Drosophila* and *C.elegans* lack kidney structures comparable to SD, the role of nephrin and Nephs homologues in junctions during *Drosophila* muscle and eye development as well as during *C.elegans* synapse differentiation may bring mechanistic insight into how these molecules could act in the mammalian SD.

In *Drosophila,* nephrin orthologues Hibris (Hbs) and Stick and stones (Sns) and Nephs orthologues Dumfounded/Kin of Irregular-chiasm-C (Duf/kirre) and Irregular-chiasm-C/Roughest (IrreC/rst) have been shown to play a role in the development of somatic muscles (Dworak *et al.,* 2001; Bour *et al.,* 2000; Ruiz-Gomez *et al.,* 2000; Strunkelnberg *et al.,* 2001). The essential part of the development is myoblast fusion in which fusion competent myoblasts aggregate around a founder cell. Hbs and Sns are expressed in fusion competent myoblasts, and Duf/kirre is expressed in founder cells. IrreC/rst is expressed in both cell types and shows homophilic adhesion activity. Hbs and Sns in turn show heterophilic adhesion activity with Duf/kirre. Therefore, their trans-interactions are suggested to be important for the myoblast fusion (Dworak, 2002). Recently nephrin was also shown to be involved in myoblast fusion in mouse and zebrafish during muscle development indicating that this pathway may be evolutionally conserved (Sohn *et al.,* 2009).

Hbs and IrreC/rst are also involved in *Drosophila* in formation of an eye unit, termed the ommatidia (Bao and Cagan, 2005). The differentiated *Drosophila* eye is composed of about 800 ommatidia in which photoreceptor cells, lens-secreting cone cells and primary pigment cells are ordered in a highly organized manner (Carthew, 2007). Hbs and IrreC/rst are expressed in different cell types and their heterophilic trans-interaction induces selective cell adhesion which is required for proper patterning of the ommatidia (Bao *et al.,* 2005).

In *C.elegans,* nephrin and Nephs orthologues SYG-2 and SYG-1, respectively, play a role in synapse formation. They have been shown to specify the localization of synapse through their trans-interaction. SYG-2 is expressed in vulval epithelium in synaptic guidepost cells and SYG-1 is expressed in hermaphrodite-specific neurons (HSNL). SYG-2 induces clustering of SYG-1 in HSNL most probably through direct interaction which consequently leads to accumulation of synaptic vesicles near SYG-1 and formation of synapse into vulval muscle and ventral cord type neurons (Shen and Bargmann, 2003; Shen *et al.,* 2004).

10. Cadherin superfamily

P-cadherin and VE-cadherin

The SD was regarded as a modified adherens junction after Reiser and co-workers localized classical type I cadherin, placental cadherin (P-cadherin), to SD (Reiser *et al.,* 2000). Later on, related atypical (type II) cadherin, vascular endothelial cadherin (VE-cadherin), was localized at the SD area (Cohen *et al.,* 2006). Both cadherins have five extracellular cadherin domains and show Ca^{2+}-dependent homophilic cell adhesion activity, although VE-cadherin shows weaker adhesion activity than P-cadherin. They are also both linked to the actin cytoskeleton via α-and β-catenins which is necessary for their full adhesion activity (Nose and Takeichi, 1986; Tanihara *et al.,* 1994; Breviario *et al.,* 1995). Deletion of P-cadherin in mouse leads to mammary gland hyperplacia and dysplacia later in life (Radice *et al.,* 1997), but no defects have been described in podocytes indicating that it is not crucial for the SD formation and maintenance, or its function is substituted by other classical cadherins. VE-cadherin deficient mice in turn die during gestation due to severe vascular defects (Gory-Faure *et al.,* 1999). Therefore, in order to know whether VE-cadherin is essential for formation of the SD, a podocyte specific knock-out mouse line should be established.

P-cadherin expression in diabetic glomeruli and developing podocytes

P-cadherin expression is decreased in glomeruli of streptozotocin-induced diabetic rats having proteinuria (Xu *et al.,* 2005), which indicates that its loss may be associated with morphological podocyte alterations observed in the model. Furthermore, injection of P-cadherin antibody into rats leads to proteinuria (Liu *et al.,* 2003). P-cadherin also forms a complex with nephrin (Lehtonen *et al.,* 2004), but its expression is not altered in patients with CNF (Ruotsalainen *et al.,* 2000). P-cadherin is expressed at higher levels during podocyte development in the S-shape body and capillary loop stage than in the mature SD (Ruotsalainen *et al.,* 2000). It has also been shown that P-cadherin and alpha- and β-catenin are expressed during podocyte development but not in mature podocytes (Yaoita *et al.,* 2002). The discrepancy of P-cadherin expression in mature podocytes (Ruotsalainen *et al.,* 2000; Yaoita *et a.,I* 2002; Xu *et al.,* 2005; Lehtonen *et al.,* 2004) may be due to differences in antibody sensitivities used in the studies, but it also indicates that cadherin/catenin expression is reduced during podocyte maturation. Collectively, based on the above P-cadherin seems not to be essential for the SD assembly, but may play a role in the modulation of the SD in certain pathological stages including diabetic nephropathy. Furthermore, since P-cadherin is able to form a complex with nephrin, it may contribute to modulation of the SD together with nephrin.

A cadherin superfamily member Fat1 regulates podocyte actin dynamics
A cadherin superfamily member Fat, which belongs to Fat cadherins, also localizes at the SD (Inoue *et al.,* 2001) and is shown to be crucial for the formation of the SD in mouse (Ciani *et al.,* 2003). Fat1 was first identified in *Drosophila* and was shown to function in cell proliferation and planar cell polarization (Bryant *et al.,* 1988; Mahoney *et al.,* 1991; Yang *et al.,* 2002). In mammals it has been shown to regulate actin dynamics via Ena/vasodilator-stimulated phosphoproteins and it plays a role in migration, adhesion and polarization of cells (Tanoue and Takeichi, 2004; Moeller *et al.,* 2004). Fat1 is a huge molecule (~500 kDa) which has an unusually large extracellular part containing 34 cadherin domains and therefore it has been speculated to serve as a spacer between adult podocyte foot processes. Similarly to P-cadherin, Fat1 is expressed at a higher level in S-shaped bodies and early capillary loop stage during podocyte development than in mature SD. In these early migrating junctional complexes Fat1 co-localizes with classical cadherins indicating that it may contribute to early junction formation together with cadherins (Yaoita *et al.,* 2005). This is consistent with the finding that Fat1 expression is down-regulated in confluent Madin-Darby canine kidney (MDCK) cells, and it has been suggested to coordinate early junction formation with cadherins (Tanoue *et al.,* 2004). The expression of Fat1 is increased in the rat PAN model in which SDs are replaced by tight-junction like structures and foot processes are effaced (Yaoita *et al.,* 2005). Taken together, Fat1-regulated actin dynamics may be important for SD assembly as well as may take part in molecular pathways leading to disruption of the SD and morphological podocyte alterations upon podocyte injury.

11. Transgenic mouse models

Transgenic mouse models are important in investigating whether a single SD protein is essential for the formation or maintence of the SD. Mouse and human podocytes share expression of most of the known SD components indicating that the results obtained from transgenic mouse models may be useful in deciphering the molecular pathways leading to SD injury and consequent proteinuria in humans. Nephrin is a good example, since deletion of the nephrin gene in mice mimics accurately the phenotype of podocytes of patients with CNF (Putaala *et al.,* 2001; Rantanen *et al.,* 2002). Since in this thesis work transgenic mouse models were used, a brief overview of the basic techniques is given below.

Transgenic technology in the mouse was developed over 30 years ago by infecting mouse embryos with viruses (Jaenisch and Mintz, 1974; Jaenisch, 1976). Later on, Gordon *et al* developed a technique to microinject DNA to the pronuclei of a fertilized mouse oocyte (Gordon *et al.,* 1980), which is still widely used. The integration of the DNA in this technique may occur during the one cell stage or for example, during the four cell stage which may cause the mouse to be mosaic for the transgene. Furthermore, since integration is random it may affect endogenous genes. The next step in developing the technique was taken when embryonic stem cells were isolated

and cultured. This allowed genetic manipulations to be done in embryonic stem cell cultures (Evans and Kaufman, 1981; Martin, 1981) followed by injection of the manipulated cells into mouse blastocysts (Gossler *et al.,* 1986). The invention of a homologous recombination technique enabled the generation of a mouse in which a specific gene is deleted or mutated (Thomas *et al.,* 1986; Thompson *et al.,* 1989).

Classical knock-out vectors have been used to show that nephrin (Putaala *et al*., 2001), podocin (Roselli *et al.,* 2004) and Neph1 (Donoviel *et al.,* 2001), for instance, are essential for the formation of the SD structure. However, this technology limits the possibility to investigate the function of podocyte proteins which are also essential for other types of cells in the body or for early developmental processes. The invention of Cre/loxP-mediated recombination solved this problem by allowing the generation of tissue-specific knockouts. In this technique one mouse line expresses a bacteriophage P1 enzyme, Cre recombinase, under the control of a tissue specific promoter. In the other mouse line the gene of interest is flanked by 34-basepair loxP sequences. When these two mouse lines are crossed, the tissue specific expression of Cre causes recombination of a loxP flanked gene in a tissue-specific manner. This technology has been used for showing that the widely expressed actin organizing protein Nck is essential for the formation of the SD and proper podocyte morphology (Jones *et al.,* 2006). To further evaluate whether single proteins are essential for a certain stage of podocyte development or mature podocyte, a technique in which Cre expression is controlled by doxycyclin (Schonig *et al.,* 2002) or tamoxifen (Metzger *et al.,* 1995) has also been developed for podocytes (Juhila *et al.,* 2006). This technology has been used to show that Nck is important for maintaining the SD structure in mature podocytes (Jones *et al.,* 2009).

Large-scale mouse mutagenesis techniques have also been developed and programmes using these techniques have been going on already over ten years which aim to establish public resources for mutant mouse lines. N-ethyl-N-nitrosourea (ENU) is a mutagen which randomly induces point mutations in a genome-wide manner. The screening is phenotype-driven and individual mutations are identified by genome-wide or regional screening (Russell *et al.,* 1979; Justice *et al.,* 1999; Hrabe de Angelis *et al.,* 2000). Gene-trap technology is in turn a technology in which specific vectors termed gene trap vectors are randomly inserted into mouse genome. The gene trap vector contains a promoterless reporter gene (lacZ) and when inserted into a gene, it causes the formation of a fusion transcript of coding sequence and a reporter gene. This leads to disruption of the gene and allows also monitoring the expression of the gene by the reporter gene (Stanford *et al.,* 2001).

12. Podocyte injury

Insights from genetic mouse models and human genetics

Podocyte foot process effacement is the most common characteristic in human glomerular diseases and in experimental animal models with proteinuria. Investigations on genetic mouse models have revealed that genetic mutations affecting proteins crucial for the SD assembly, podocyte-GBM connection, podocyte actin cytoskeleton organization, and proper apical domain composition of podocytes can lead to foot process effacement and proteinuria. The crucial SD proteins in mouse have been shown not only to serve as structural proteins for the SD but also to participate in molecular pathways leading to actin cytoskeleton organization, polarization and anti-apoptotic signalling. They include both transmembrane proteins bridging across the SD as well as cytosolic adapter proteins tethering the transmembrane proteins to the actin cytoskeleton and signalling pathways. Lack of some of these proteins leads to heavy proteinuria already at birth (nephrin, podocin) whereas in some other cases (Fyn, α-actinin-4) the deficiency leads to proteinuria later in life indicating that these proteins would rather play a role in maintaining the integrity of the SD (Michaud *et al.,* 2007) (see Table 1 for summary). Some of these crucial SD proteins in the mouse have also been associated with human glomerular diseases of which nephrin is the most famous, since lack of it causes severe proteinuria and consequent death without kidney transplantation (Kestila *et al.,* 1998). Podocin is associated with autosomal recessive steroid-resistant nephrotic syndrome in which proteinuria starts also at childhood (Boute *et al.,* 2000). Similarly as in the mouse, α-actinin-4 is associated with late onset of proteinuria in FSGS (Kaplan *et al.,* 2000). (Table 2).

Table 1: *Deletion of genes encoding essential SD proteins and their binding partners in the mouse.*

Gene disruption	Subcellular localization	Onset of proteinuria	Viability	Function	Reference
nephrin	transmembrane, IgG superfamily	within 24 hours	die within 24 hours	actin organization, anti-apoptotic, Ca^{2+} signalling	(Rantanen *et al.*, 2002; Putaala *et al.*, 2001)
Neph1	transmembrane, IgG superfamily	within 1-3 days	die within 1-12 days	actin organization	(Donoviel *et al.*, 2001)
Fat1	transmembrane, Fat cadherins	not determined	die within 48 hours	actin organization	(Ciani *et al.*, 2003)
podocin	hairpin-like integral membrane protein, Stomatin superfamily	within 24 hours	die within 5 weeks	signalling	(Roselli *et al.*, 2004)
Fyn	cytosolic, Src family kinase	within 3-4 months	die within 60 weeks	signalling	(Yu *et al.*, 2001)
alpha-actinin-4	cytosolic, superfamily of actin-binding proteins	later in life (not specified)	some die during perinatal period, others survive till later in life	actin cross-linking, tethering transmembrane proteins to actin	(Dandapani *et al.*, 2007; Kos *et al.*, 2003)
aPKC	evolutionary conserved polarity protein	at 4 weeks	die within 4-5 weeks of age	polarization	(Huber *et al.*, 2009)
CD2AP	cytosolic adapter protein	shortly after birth (not specified)	die at 6 weeks of age	anti-apoptotic, actin organization	(Shih *et al.*, 1999)
Nck1 Nck2	SH2/3 domain containing adapter protein	at birth	later in life (not specified)	actin organization	(Jones *et al.*, 2006)

Table 2: *Mutated genes encoding SD proteins and their binding partners causing human nephrotic syndromes*

Mutated genes	Protein family	Syndromes	References
Early onset:			
CD2AP	cytosolic adapter protein	focal and segmental glomerular sclerosis	(Kim *et al.,* 2003)
Nephrin	IgG superfamily	congenital nephrotic syndrome of the Finnish type	(Kestila *et al.,* 1998)
Podocin	stomatin superfamily	autosomal recessive steroid-resistant nephrotic syndrome	(Boute *et al.,* 2000)
Late onset:			
α-actinin-4	cytosolic, supefamily of actin-binding protein	familial focal and segmental glomerular sclerosis	(Kaplan *et al.,* 2000)
TRCP6	canonical transient receptor potential subfamily	focal and segmental glomerular sclerosis	(Winn *et al.,* 2005; Reiser *et al.,* 2005)

Insights from experimental rodent models

The genetic mouse models provide information about molecular mechanisms which are important to either proper SD assembly or maintenance of the integrity of the SD. However, the drastic phenotypes of the mice rarely mimic the human glomerular diseases even though there are exceptions like CNF. In most cases the development of proteinuria in glomerular diseases is suggested to be a combination of environmental and genetic factors. Therefore, characterizing rodent models in which proteinuria is induced by challenging glomerular filtration barrier in different ways as well as investigating biopsies obtained from patients with acquired glomerular diseases is essential to further understand the pathophysiological mechanisms of glomerular diseases.

Podocyte injury can be manifested in the mouse or rat by injections of toxic substances including adriamycin (adrimycin nephrosis in mouse) or puromycin aminonucleoside (PAN model in rat) or by challenging them with excess amount of albumin (albumin overload model in mouse and rat) (Table 3). In some of the models the morphological findings share close similarities with human diseases. The PAN model in rat has been suggested to resemble MCD in humans (Messina *et al.,* 1987), adriamycin nephrosis in turn shares characteristics with FSGS (Chen *et al.,* 1998) and some of the pathological findings from passive Heymann nephritis in the rat are similar to human membranous nephropathy (Pippin *et al.,* 2009). However, the comparison between rodent models and human diseases should not be over-simplified.

Many of the models share similar characteristics including foot process effacement (Figure 4) and formation of tight junctions between the foot processes. In some models like protamine sulphate, these alterations occur already 10 minutes after treatment

and are reversible, whereas in other models (adriamycin and PAN model) the development of podocyte injury takes even one week. Some of the well-characterized models work only with the rat (PAN and protamine sulphate models) and many of the models are very strain-dependent. Genetic deletions are usually made in mouse, which excludes the possibility to study the role of a single gene during development of podocyte injury for example in well-characterized PAN or/and protamine sulphate models (Pippin *et al.,* 2009) that can be used only for rats.

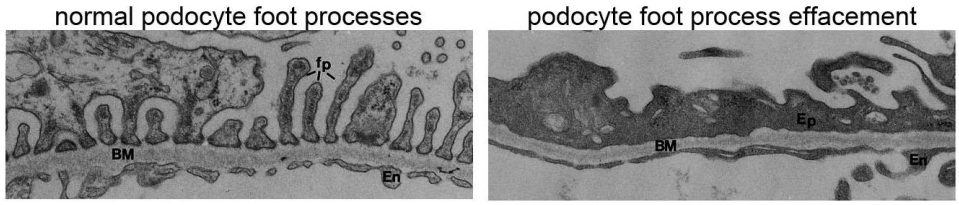

Figure 4: *Podocyte foot process effacement in PAN model. The podocyte foot process effacement is characterized by broadening of the foot processes as well as a decreased number of podocyte foot processes and filtration slits. BM, glomerular basement membrane; En, glomerular endothelial cells; fp, normal podocyte foot processes and Ep, effaced podocyte foot process. Modified from (Takeda et al., 2001).*

Table 3: *Inducible rodent models with proteinuria*

Animal model	Mechanisms	Species	Onset of proteinuria	Podocyte phenotype	References
Protamine sulphate model	altering anionic charges of the glomerular filter	rat	40-70 minutes	Rapid reversible foot process effacement (10-15 minutes after treatment), formation of tight junctions, apical displacement of SDs	(Seiler *et al.,* 1975; Kurihara *et al.,* 1992)
Lipopolysaccari-de model	Immunological	mouse	24-72 hours	Foot process effacement (within 24 hours)	(Reiser *et al.,* 2004)
Protein/ albumin overload	increasing filtration rate by excess amount of albumin	rat, mouse	6-24 hours (repeatable injections of albumin to obtain continuous proteinuria)	Foot process effacement, detachment of podocytes from the GBM and accumulation of pinocytic vesicles, protein absorption droplets and vacuoles	(Anderson and Recant, 1962; Davies *et al.,* 1985; Weening *et al.,* 1987)
Passive Heymann nephritis	immunological (accumulation of immune deposits)	rat	4-6 days	Foot process effacement, formation of tight junctions, detachment of podocytes from the GBM and loss of SDs	(Heymann *et al.,* 1959; Kerjaschki *et al.,* 1987; Schneeberger *and Grupe,* 1976)
Adriamycin nephrosis	toxic	rat, mouse	5-7 days	Foot process effacement and fusion	(Wang *et al.,* 2000)
PAN nephrosis	toxic	rat	4-6 days	Foot process effacement, formation of tight junctions, apical displacement of SDs, detachment of podocytes from the GBM and accumulation of protein absorption droplets and vacuoles	(Caulfield *et al.,* 1976; Messina *et al.,* 1987; Kurihara *et al.,* 1992)

AIMS OF THE PRESENT STUDY

The SD is an important structure for glomerular filtration function, since lack of or mutations in many SD components result in severe glomerular diseases. Nephrin is one of the essential SD components since without nephrin SDs are not formed resulting in massive proteinuria and severe nephrotic syndrome, CNF. However, detailed molecular mechanisms of how nephrin participates in the formation of this specialized cell-cell contact, the SD, are largely unknown. This thesis work has aimed at investigating the nephrin protein complex and its role in the formation and maintenance of the SD.

The specific aims of this thesis work were the following:

I. To identify novel proteins belonging to nephrin protein complex in order to define the essential molecular complex required for the establishment of the SD.

II. To gain novel insights of how nephrin and its binding partners may function in the formation of the SD.

III. To investigate the expression and role of nephrin associating proteins in injured podocytes.

MATERIALS AND METHODS

1. Clinical samples

Human kidney samples were used for immunofluorescence, reverse transcriptase-polymerase chain reaction (RT-PCR), immunoblotting and protein interaction investigations as described below. Kidney samples were obtained from CNF patients and cadaver donors unsuitable for transplantation due to vascular anatomic reasons. Normal human brain sample was received during surgery next to tumour tissue in temporal lobe from a young adult (Department of Surgery, University of Helsinki). All tissues were frozen in liquid nitrogen and stored at -70 ˚C (Study I).

2. Animals

Nephrin deficient mouse line
Nephrin TRAP mice were established in the GSF Center for Environment and Health, Institute of Mammalian Genetics (Neuherberg, Germany) (Hill and Wurst, 1993) and characterized in by Rantanen and colleaques (2002; Study IV).

Generation of inducible podocyte specific **β**-catenin knock-out mise
Podocyte-specific doxycycline-inducible Cre Recombinase construct was generated by using the core construct which has been previously published (Utomo *et al.,* 1999) and has been described earlier in (Juhila *et al.,* 2006). Briefly, podocin promoter was cloned upstream of a gene encoding transcription factor reverse tetracycline-controlled transcriptional activator (rtTA) into the core construct containing rtTA-inducible promoter upstream a gene encoding Cre recombinase. The transgene was released from the backbone vector by restriction, purified and injected into fertilized oocytes of FVB/N mice. Genotyping of transgenic mice was performed with PCR using primers recognizing *Cre* (5'-gaccaggttcgttcactca-3' and 5'-tagcgccgtaaatcaat-3'). A transgenic mouse line containing *loxP* sites which flank β-catenin gene in B6.129 mouse strain was purchased from Jackson Laboratories (The Jackson Laboratories, Bar Harbor, ME). Podocyte-specific Cre mouse line was backcrossed for five generations and β-catenin floxed mouse line for 10 generation to C57BL/6J mouse strain. Bitransgenic mice were identified by PCR using primers detecting *Cre* (see above) and floxed β-catenin gene (5'-aaggtagagtgatgaaagttgtt-3' and 5'-caccatgtcctctgtctattc-3'). At the age of 8 weeks the expression of Cre recombinase was induced by administration of 1 mg/ml of doxycycline supplemented with sucrose in drinking water for 2 weeks. β-catenin gene deletion was detected by PCR using 5'-aatcacagggacttccataccag-3' and 5'-gcccagccttagcccaact-3'primers (Study III).

Adriamycin treatment

Doxycyclin-treated β-catenin deficient mice (β-cat$^{fl/fl}$/Cre) and their controls carrying Cre recombinase (β-cat$^{wt/wt}$/Cre) were tail vein-injected adriamycin. To measure urinary albumin excretion, 24h urine was collected in metabolic cages and urinary mouse albumin was measured at 3 and 6 six days after adriamycin treatment by enzyme-linked immunosorbent assay (Study III).

3. Cell lines

Different cell lines were used in this thesis work to investigate expression, protein-protein interactions and function of nephrin and its associating proteins.

Table 4: *Summary of the cell lines used in the studies*

Cell line	Description	Culture medium	Supplier/ reference	Used in
Human podocytes	Primary culture of human podocytes	Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum, 2.5 mM glutamine, 0.1 mM sodium pyruate, 5 mM HEPES, 100 U/ml penicillin, 0,1 mg/ml streptomycin, 2 mM glutamine, insulin, transferrin and 5 mM sodium selenite.	(Greiber *et al.,* 1998)	I
HEK 293	Human embryonic kidney cell line	DMEM (1000 mg glucose/l) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, 0,1 mg/ml streptomycin and 2 mM glutamine	ATCC	II
Mouse podocyte cell line	Conditionally immortalized cells containing the temperature-sensitive SV40 large T antigen	RPMI 1640 medium supplemented with 100 U/ml penicillin and 0,1 mg/ml streptomycin	(Schiwek *et al.,* 2004)	II
293T	Highly transfectable derivative of HEK 293 cell line	DMEM (4500 mg glucose/l) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, 0,1 mg/ml streptomycin, 2 mM glutamine and 25 mM Hepes	ATCC	IV
L-cells	Mouse L fibroblasts (L-929)	DMEM (4500 mg glucose/l) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, 0,1 mg/ml streptomycin, 2 mM glutamine and 25 mM Hepes	ATCC	IV

4. Primary antibodies

The used antibodies are decribed in the original publications (Study I-III) and in the submitted manuscript (Study IV).

5. Constructs

Full-length and different parts of nephrin and its associating proteins were subcloned to mammalian and bacterial vectors (Table 5). The produced fusion proteins were used for protein-protein interaction assays and functional cell assays.

Table 5: *Summary of the constructs used in the studies*

Protein	Species	Description	Vector (supplier)	Reference	Used in
densin	human	full-length	pRK5-myc	(Izawa *et al.*, 2002)	II
densin	mouse	full-length	pEGFP-N1 (Clontech laboratories)		II
densin	human	C-terminal fragment (amino acids 1242-1537) and its truncations	pGEX-6P-2 (Amersham Biosciences)		II
EGFP	jellyfish	full-length	pMSCVpuro (Clontech laboratories)		IV
Neph3	mouse	full-length	pMSCVpuro (Clontech laboratories)		IV
Neph3	mouse	full-length	pcDNA3.1/myc/his (InVitrogen)		IV
Neph3	human	extracellular domain	signal plgplus (R&D Systems)		IV
Neph3	human	intracellular domain	derivative of pCDM8 vector	(Tsiokas *et al.*, 1997)	IV
Neph1	mouse	full-length	p-Babe-hygro	(Morgenstern and Land, 1990)	IV
Neph1	mouse	intracellular domain	derivative of pCDM8 vector	(Tsiokas *et al.*, 1997)	IV
nephrin	rat	full-length	pcDNA3.1/myc/his (InVitrogen)		IV
nephrin	rat	full-length	pMSCVneo (Clontech laboratories)		IV
nephrin	human	intracellular domain	derivative of pCDM8 vector	(Tsiokas *et al.*, 1997)	IV

6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from kidney and brain tissues as wells as from human podocytes using Trizol reagent. Genomic DNA was removed by DNase I treatment in the presence of human RNase inhibitor and cDNA was synthesized with the oligo dT15-primers and Moloney murine leukemia virus reverse transcriptase (M-MLV RT) in the presence of RNase inhibitor. PCR was performed using densin-specific primers (5'atgctttccctgacaactgg3' and 5'gtgtgtctgtgggtggactg3') and verified with another primer pair (5'gacaagccatcagataaca3' and 5'agttgcactcgaatgacag3') (Study I).

7. Retroviral infection and establishment of stable cell lines

Retroviral constructs (Neph3-EGFP-pMSCVpuro, EGFP-pMSCVpuro or nephrin-pMSCVneo) were co-transfected with packaging vector PKAT (Finer *et al*., 1994) into 293T cells. Alternatively, retroviruses were produced by transfecting Phoenix Ampho packaging cell line (from Garry Nolan, Stanford University, Stanford, CA) with retroviral vector (Neph1-pBabe-hygro or pBabe-hygro). L-cells were infected with retroviral supernatants and puromycin, G418 and hygromycin were used to select for cells stably expressing nephrin, Neph1, Neph3 or EGFP.

8. Cell assays

Calcium switch assay

Cultured mouse podocytes were grown to confluence on coverslips and washed rapidly twice with calcium- and magnesium-free (Ca^{2+}/Mg^{2+}-free) Hanks balanced salt solution (HBSS). After that cells were first incubated in Ca^{2+}/Mg^{2+}-free HBSS for 20 min at 38 ºC and then in normal culture medium (RPMI 1640) containing calcium for 100 min at 38 ºC. HBSS containing calcium was used as a control in the experiments. Cells were fixed after 2 and 20 min without calcium and after 20 and 100 min in calcium containing medium for immunostaining (Study II).

Puromycin aminonucleoside model on cells

Confluent cultured mouse podocytes were maintained in culture medium supplemented with 100 µg/ml puromycin aminonucleoside for 24 h. Cells were fixed, immunostained and analyzed by immunofluorescence microscopy (Study II).

Hanging drop assay

Equal amounts of L-cells plated on the previous day were trypsinized, suspended in normal culture medium and drops of single cell suspension were placed on culture dish lids filled with PBS to avoid drying of the drops. After incubating the hanging drops 24 hours in a cell incubator (N_{24}), the cells in the hanging drops were gently pipeted up and down or they were trypsinized (N_T). After that the cells were fixed with glutaraldehyde, photographed by phase contrast microscope and the particles were counted. More than four cells in a cell aggregate was determined as a single particle. Total particle

number was calculated at each time point and aggregation index was calculated using the formula $(N_T-N_{24})/N_T$. Student t test was used for statistics (Study IV).

9. Protein interaction studies

Co-immunoprecipitation assay on cell lysates
HEK 293 or 293T cells were transfected with desired plasmids and after about 48 hours cells were lysed on ice in lysis buffer supplemented with protease and phosphatase inhibitors. Insoluble material was removed by centrifugation and total protein concentration was measured. Cell lysates were pre-cleared with protein A or G sepharose followed by overnight incubation with primary antibodies or control IgGs. Protein A or G sepharose were used to capture antibody-protein complexes and unbound proteins were washed off with lysis buffer. Samples were boiled in Laemmli sample buffer and immunoblotted (Study II and IV).

Production of densin GST fusion proteins
C-terminal fragments of human densin were cloned into pGEX-6P-2 vector. Constructs were transformed into BL21 or DH5alpha and the expression of the fusion proteins was induced by isopropyl β-D-thiogalactosidase (IPTG). The fusion proteins were purified with gluthathione-sepharose beads and analyzed by SDS-PAGE and Coomassie staining (Study II).

Production of the extracellular domain of Neph3
The extracellular domain of human Neph3 was cloned into a signal plgplus vector which contains the signal sequence of CD33 and the FC domain of human IgG1 and is used for secreted expression in mammalian cells. The construct was transfected into 293T cells and after 48 hours, the culture medium was harvested, the recombinant protein was captured using protein A sepharose and unbound proteins were washed away. The purified protein and control protein containing only human IgG1 were analyzed by SDS-PAGE and Coomassie staining (Study IV).

Pull-down assays
Human or rat glomeruli were isolated by cutting cortical kidney tissue into small pieces and passing it through series of sieves of decreasing pore sizes (250 µm, 150 µm and 75 µm). Glomeruli were collected from the final collection sieve and the purity of the glomerular fraction was analyzed by microscopy. Glomeruli were lyzed on ice with glass-ware homogenizer in lysis buffer, the debris was removed by centrifugation, total protein concentration was measured and lysate was incubated with recombinant proteins conjugated to glutathione or protein A sepharose beads. Beads were washed with lysis buffer, boiled in Laemmli sample buffer and the samples were analyzed by immunoblotting (Study II and IV).

10. Immunoblotting

Proteins were separated by SDS-PAGE gels, electrotransferred to nitrocellulose or polyvinylidene difluoride (PVDF) membranes and blocked with blocking buffer. Membranes were incubated with primary antibodies, washed and the bound antibodies were detected using secondary antibodies conjugated with horseradish peroxidase or fluorescent dye (Study I-IV).

11. Immunofluorescence microscopy

Immunofluoresence microscopy on kidney tissues
Frozen kidney cortical tissue sections were fixed with PFA (followed by permeabilization with TritonX-100) or acetone. After PBS washes, sections were blocked with blocking solution and incubated with primary antibodies overnight. The following day sections were washed with PBS, incubated with fluorescently labelled secondary antibody followed by washing with PBS and mounting (Study I, III and IV).

Immunofluoresence microscopy on cultured cells
Cells on glass coverslips were washed with PBS and fixed with acetone or PFA (followed by permeabilization with TritonX-100). After PBS washes cells were blocked in the blocking solution, incubated with primary antibodies and washed. Antibody binding was visualized by fluorescently labelled secondary antibody and F-actin was detected by incubating cells with fluorocrome-conjugated phalloidin. Finally cells were washed and mounted in Mowiol (Study II and IV).

All specimens were examined by conventional or confocal microscopes.

Surface staining
Cells cultured on glass coverslips were incubated on ice with an antibody directed against the extracellular domain of nephrin (#033) followed by washes. Stained cells were fixed PFA at RT, blocked in the blocking solution and incubated with fluorescently labelled secondary antibody. After washes cells were mounted in Mowiol and viewed under confocal microscope (Study IV).

12. Immunohistochemistry

Kidneys were fixed with PFA, embedded in paraffin and cut into sections. Sections were first deparaffinized in xylene and then rehydrated through a graded series of ethanol (100% to 50%). For antigen retrieval, sections were heated in 10 mM citrate buffer pH 6 in microwave. Endogenous peroxidase activity was quenched with hydrogen peroxide followed by blocking and incubation with primary antibodies overnight. On the following day, sections were incubated with peroxidase-conjugated secondary antibodies and the peroxidase reaction was developed with AEC substrate. At the end, the sections were counterstained with hematoxylin, mounted and examined by light microscope (Study III).

13. Electron and immunoelectron microscopy

Electron microscopy
Mouse kidney cortices were fixed in glutaraldehyde and postfixed in osmium tetroxide (OsO_4). After fixing the cortices were stained en-block in uranyl acetate, dehydrated in ethanol and embedded in LX112. Thin sections were incubated with uranyl acetate and lead citrate and viewed with a JEM-1400 Transmission Electron Microscope (Study III).

Immunoelectron microscopy
Rat kidney cortices were fixed with formaldehyde followed by embedding in Lowicryl K4M. Ultrathin sections were blocked with in the blocking solution and incubated with anti-densin antibody followed by anti-rabbit gold conjugate (Study I).

Quantification of podocyte foot process effacement
The number of podocyte foot processes per μm of GBM in mouse glomeruli was measured from electron micrographs using ImageJ-program which was calibrated by the marker bar. Four to five random glomeruli from each mouse were measured. From each glomerulus the length of five random capillaries was measured and the number of podocyte foot processes was counted manually. The results were presented as the number of foot processes per μm GBM length. Student *t* test was used for statistical analysis (Study III).

14. Ethical issues

The human kidney sample collection and procedures decribed herein were approved by the Ethics committees' of the Hospital for Children and Adolescent, University of Helsinki. The animal experiments were approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland.

RESULTS

1. Identification of densin as part of the nephrin protein complex

To investigate the role of nephrin in the formation of the SD, we searched for novel components of the nephrin protein complex. To this end, we performed immunoprecipitation assays on rat glomerular lysates using an anti-nephrin antibody. The immunoprecipitates were separated by SDS-PAGE and silver stained. One distinct protein band at about 200 kDa was cut out, digested with trypsin and the peptides were identified by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry. Eleven peptides matched with the rat densin protein sequence. Densin has been originally identified from the post-synaptic densities of rat forebrain in which it associates with N-methyl-D-aspartate (NMDA) receptor complexes (Apperson *et al.,* 1996; Strack *et al.,* 2000; Ohtakara *et al.,* 2002; Izawa *et al.,* 2002). To confirm the association of densin with nephrin in podocytes, co-immunoprecipitation assay was performed on human glomerular lysates. Immunoblotting with anti-densin antibody showed that nephrin precipitated densin (Study I, Figure 3).

2. Densin localizes to the glomerular slit diaphragm

To further confirm that densin is expressed in podocytes, we performed RT-PCR and immunoblotting assays. RT-PCR analysis showed that densin mRNA was expressed in human kidney cortical tissue, human glomeruli and human cultured podocytes by using densin-specific primers (Study I, Figure 2). Immunoblotting analysis showed that densin protein was expressed in both human glomeruli and cultured human podocytes (Study I, Figure 3). However, in human glomeruli densin appeared as a 210 kDa band, whereas in brain and cultured podocytes the molecular weight of densin was 185 kDa. The difference may be explained by different post-translational modifications including glycosylation. These results indicate that densin is expressed in podocytes in human glomeruli. To further investigate the localization of densin in kidney immunofluorescence and immunoelectron microscopy analysis were performed. Immunofluorescence stainings showed podocyte-like staining pattern for densin and it was also detected in the brush border of proximal tubuli (Study I, Figure 5). Immunogold labelling revealed that densin localized within the SD (Figure 6) (Study I, Figure 6). The subcellular localization of densin was investigated by over-expressing EGFP-tagged densin in cultured mouse podocytes. Immunofluorescence analysis showed that densin was exclusively localized in the cell-cell contacts and it was not found along free membrane edges. In the cell-cell contacts it co-localized with F-actin (Study II, Figure 3) and with nephrin (data not shown). These results confirm that densin is expressed in podocytes and suggest that it associates with cell adhesion protein complexes at the SD.

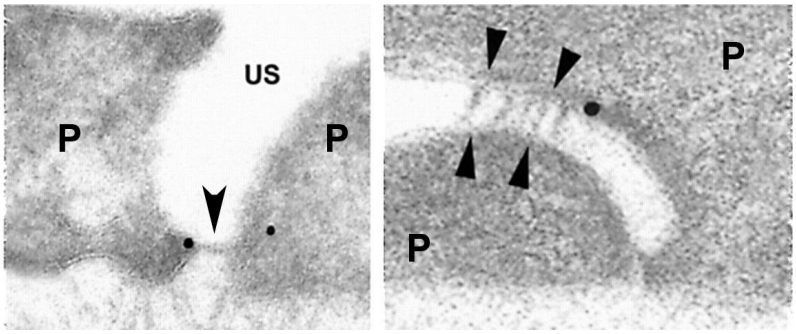

Figure 6: *Densin localizes to the SD. The arrowheads point to the SDs. P, podocyte and US, urinary space. Modified after (Study I, Figure 6).*

3. Densin interacts with β-catenin

In order to investigate the function of densin in podocytes, we searched interaction partners for densin by the yeast-two-hybrid method using the C-terminal domain of human densin as a bait. The screening yielded three distinct clones encoding β-catenin, two clones of plakophilin/p0071 and one clone of alpha-actinin-2. Of these, β-catenin was chosen for further investigations, since plakophilin/p007 and alpha-actinin-2 are homologous with delta-catenin and alpha-actinin-4, which are known to interact with densin in post-synaptic densities (Izawa *et al*., 2002; Walikonis *et al*., 2001). The three clones of β-catenin encoded the C-terminal fragment of β-catenin containing at least one armadillo repeat and PDZ-binding motif (Study II, Figure 1). The interaction between densin and β-catenin was confirmed by reciprocal co-immunoprecipitation assay on myc-tagged densin transfected into HEK 293 cells. Densin-myc was able to precipitate endogenous β-catenin and densin-myc was detected in β-catenin precipitates (Study II, Figure 1). The binding domain in densin for β-catenin was determined by GST-pull-down assays on human glomerular lysates. To this end several GST-fusion proteins of the C-terminal fragment of densin were produced. The pull down assays with densin fusion proteins revealed that a short region downstream of the transmembrane region of densin (aa 1242-1360) was able to bind to β-catenin, but not the PDZ-domain. Since the whole C-terminal fragment had stronger affinity against β-catenin, other parts of the C-terminal fragment most likely were involved in the binding or/and proper folding of the binding site. Finally, densin and β-catenin distribution was investigated in cultured mouse podocytes by immunofluorescence microscopy. EGFP-tagged over-expressed densin co-localized with endogenous β-catenin at the cell-cell contacts in mouse podocytes further supporting their interaction (Study II, Figure 3). These data show that densin binds to adherens junction protein β-catenin and, therefore, suggest that it may play a role in the formation of cell-cell contacts.

4. Densin behaves in a similar fashion as adherens junction proteins in cell junctions

To investigate whether densin acts in a similar manner as adherens junction proteins, calcium switch assay was performed on densin-EGFP transfected mouse podocytes. Calcium switch assay takes advantage the calcium-dependent trans-interaction of cadherins, since calcium depletion results in disruption of the adherens junctions and translocation of cadherin/catenin complex to cytoplasm. After calcium is added to the cells, the reformation of the cell-cell contacts can be investigated from early cadherin/catenin clustering into puncta when they are starting to be anchored to actin cytoskeleton till they fuse and mature to form continuous belt-like junctions with strong intercellular adhesion (Adams *et al.,* 1998). After two minutes without calcium, podocytes started to lose their mature cell-cell contacts. At this time point most of densin-EGFP and β-catenin were found at the plasma membrane, but some of them were already detected in cytoplasm (Figure 7) where they co-localized. After 20 minutes, when all the cell-cell contacts were lost, densin and β-catenin co-localized in cytoplasm in a dot-like pattern. Twenty minutes after addition of normal culture medium immature junctions were visible and 100 minutes after belt-like mature junctions were formed. Densin and β-catenin co-localized both in the immature and mature junctions (Study II, Figure 4). These results further confirm that densin binds to β-catenin and suggest that it may play a role in the formation of adherens junctions.

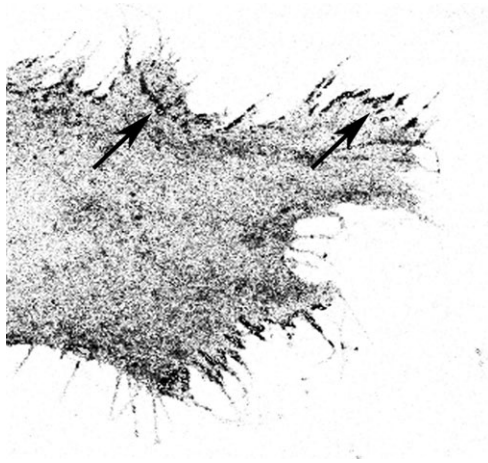

Figure 7: *After calcium depletion densin is trans-located from the plasma membrane into the cytoplasm in cultured mouse podocytes (arrows).*

Injection of puromycin aminonucleoside (PA) into rats results in podocyte injury and development of proteinuria (Caulfield et al., 1976; Messina et al., 1987; Kurihara et al., 1992). Similarly, the PA treatment has been shown to cause injury to cultured podocytes by resulting in disruption of cell-cell contacts and reorganization of actin cytoskeleton (Rico *et al.*, 2005). Therefore, we used the PA model on cultured mouse podocytes to investigate the behaviour of densin and β-catenin upon injury-induced junction disruption. Incubation of podocytes in 100 µg/ml of PA for 24 hours resulted in disruption of cell junctions associated with translocalization of densin and beta-catenin into cytoplasm. Similarly as in calcium switch assay, they co-localized in cytoplasm in a dot-like pattern after the PA treatment (Study II, Figure 5). These results further confirm that densin functions in β-catenin protein complexes.

5. Densin is up-regulated in kidneys of CNF patients

Lack of nephrin in CNF patients results in loss of SDs and formation of tight-junction like structures between podocytes (Lahdenkari *et al.*, 2004). Since densin forms a complex with nephrin and adherens junction proteins, we investigated whether the expression of densin is altered in kidneys of CNF patients. Semi-quatitative RT-PCR analysis showed that densin mRNA levels were increased in kidney cortices of CNF patients compared to normal kidney cortices. Similarly, densin protein levels were increased in glomeruli of CNF patients (Study I, Figure 4) compared to normal glomeruli. However, semiquantitative immunofluorescence analysis showed decreased staining in glomeruli of CNF patients compared to controls (Study I, Table 1). These results suggest that densin may play a role in the formation of tight-junction like structures in the podocytes of CNF patients.

6. β-catenin is dispensable for adult mouse podocyte

To investigate the *in vivo* function of β-catenin in podocytes, we established a mouse model in which we were able to silence β-catenin in adult mouse podocytes by doxycycline inducible Cre-loxP system. Lack of β -catenin DNA fragment (exon 2 to 6) after doxycyclin treatment was confirmed by RT-PCR (Study III, Figure 1). β-catenin deficiency did not lead to albuminuria (data not shown) and light microscopy investigations on hematoxylin-eosin stained tissue sections showed no obvious morphological changes in the kidney (Study III, Figure 2). Immunofluorescence stainings revealed no alterations in the expression of SD proteins including cadherins, podocin, nephrin and ZO-1 in glomeruli (Study III, Figure 3). The expression of nephrin and podocin was further confirmed by semiquantitative immunoblotting, which similarly showed no alterations between β-catenin deficient and control mouse kidneys (Study III, Figure 3). These data show that β-catenin is not essential for maintaining the SD in adult mouse podocytes.

7. β-catenin promotes adriamycin-induced podocyte injury

Adriamycin treatment in mouse results in podocyte injury including podocyte effacement and loss of SDs in association with albuminuria (Wang *et al.,* 2000). To investigate whether β-catenin plays a role in adriamycin nephropathy, we injected adriamycin (13 mg/kg of body weight) into tail-vein of β-catenin deficient and control Cre mice. Three days after injection control mice developed significant ($p \leq 0.05$) albuminuria which stayed significant six days post-injection. In contrast, β-catenin deficient mice did not show significant albuminuria compared to the level of albumin in urine before the adriamycin treatment. (Study III, Figure 4). The increased albuminuria in control mice was associated with lower well-being score already three days after injection. The well-being score showed significant difference between the genotypes for six days after the injection (Study III, Figure 4). These data suggest that β-catenin plays a role in the development of albuminuria in adriamycin nephropathy in mouse.

Since loss of β-catenin in podocytes results in albuminuria, we investigated whether β-catenin deficiency protects podocyte foot processes against effacement. To this end, we performed morphometric measurements from electron micrographs and showed that control mice showed significantly ($p \leq 0.001$) increased podocyte foot process effacement (1.3±0.2 foot process per µm of GBM) compared to β-catenin deficient mice (1.7±0.3 foot process per µm of GBM). The increased podocyte foot process effacement was associated with disruption and dislocalization of the SD towards the apical aspect of podocyte. However, podocyte foot processes with normal morphology and proper SDs could also be found in control mice as well as effaced podocytes were found in β-catenin deficient mouse glomeruli although less frequently (Study III, Figure 5). These data suggest that β-catenin plays a role in adriamycin-induced podocyte foot process effacement (Figure 8).

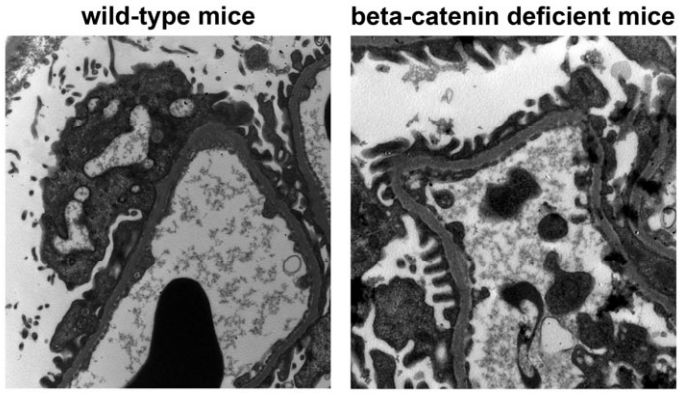

Figure 8: *β-catenin deficient mice show lower level of podocyte foot process effacement compared to wild-type control mice after adriamycin treatment.*

8. Neph3 is a component of nephrin-Neph1 protein complex

Neph3 localizes to the SD and shares high homology with Neph1 and Neph2 (Ihalmo *et al* 2003; Ihalmo *et al* 2007), which bind to nephrin (Gerke *et al.,* 2003; Gerke *et al.,* 2005; Barletta *et al.,* 2003; Liu *et al.,* 2003). We therefore set out to investigate whether Neph3 is able to bind to nephrin. To this end, we co-transfected constructs encoding full-length nephrin and myc-tagged Neph3 into 293T cells and performed reciprocal co-immunoprecipitation assays using anti-nephrin and anti-myc antibodies. The results showed that nephrin was able to precipitate Neph3-myc and reciprocally, Neph3-myc precipitated nephrin (Study IV, Figure 1). These results show that Neph3 behaves in a similar fashion as Neph1 and Neph2 by binding to nephrin.

The extracellular domains of Neph1 and nephrin form heterodimers which have been suggested to bridge opposite podocyte foot processes and participate in the formation of the SD (Gerke *et al.,* 2003; Barletta *et al.,* 2003; Liu *et al.,* 2003). To investigate whether the extracellular domains of Neph3 and nephrin bind to each other, the construct encoding extracellular domain of Neph3 fused to human IgG Fc and CD33 signal sequence was produced. The recombinant protein was produced in 293T cells and purified from the cell culture medium. The purified extracellular part of Neph3 was used for pull-down assays on rat glomerular lysates. The results showed that nephrin and podocin were pulled down with the extracellular part of Neph3 from rat glomerular lysates (Study IV, Figure 1) and indicate that the extracellular parts of nephrin and Neph3 bind to each other.

To test whether the intracellular domains of nephrin, Neph1 and Neph3 are able to interact with Neph3, we used constructs in which the C-terminal domains of the proteins were fused to the leader sequence of CD5, CH_2 and CH_3 domains of human IgG1 and transmembrane domain of CD7. The intracellular constructs were co-transfected with full-length Neph3-myc into 293T cells and the intracellular constructs were pulled down using protein G-sepharose. The results showed that the intracellular domains of nephrin, Neph1 and Neph3 were all able to precipitate full-length Neph3-myc (Study IV, Figure 1). These results show that Neph3 forms homodimers and heterodimers with nephrin and Neph1 via its intracellular domain.

Collectively, these results indicate that Neph3, Neph1 and nephrin may form a functional complex needed for the SD assembly.

9. Neph1 and Neph3 show homophilic and heterophilic adhesion activity with nephrin

To investigate whether the interactions between nephrin and Neph3 or nephrin and Neph1 induce cell adhesion, we used mouse L fibroblasts (L-cells) as an *in vitro* model to evaluate their adhesion activity. L-cells lack expression of adherens junction proteins and consequently they cannot form proper cell junctions and have been widely used to investigate adhesion activity of several proteins (Nagafuchi *et al.,* 1987; Satoh-Horikawa *et al.,* 2000). To investigate the adhesion activity of nephrin, Neph1 and Neph3, we produced stable cell lines expressing nephrin, EGFP-tagged Neph3, or HA-tagged Neph1 alone or in combination using retroviruses and antibiotics selection (G418 for nephrin, puromycin for Neph3-EGFP and EGFP, and hygromycin for Neph1-HA). The expression of the proteins in L-cells was analyzed by immunoblotting which showed that nephrin expressed in L-cells had similar molecular weight as endogenous nephrin in rat glomerular lysates. Equally, Neph3-EGFP and Neph1-HA expressed in L-cells migrated at their expected molecular weights. Localization of the over-expressed proteins was investigated by immunofluorescence stainings showing that nephrin localized mainly in free membrane edges and surface staining showed that it was present at the plasma membrane in L-cells. Neph3-EGFP and Neph1-HA in turn localized mainly in cell-cell contacts (Study IV, Figure 2). These results show that nephrin, Neph1 and Neph3 are localized at the plasma membrane and are similarly glycosylated as endogenous proteins in podocytes suggesting that L-cells provide a proper model system to investigate their adhesive properties.

To investigate whether nephrin, Neph1 or Neph3 can form cell-cell contacts alone or only after binding to each other either in cis or trans configuration, a hanging drop assay was used. L-cells expressing nephrin showed an aggregation index comparable to L-EGFP cells indicating that nephrin cannot alone form cell-cell contacts. Similarly, L-cells expressing nephrin and Neph1 (L-nephrin-Neph1-HA) in the same cells did not reach a significant increase in aggregation index compared to control cells (L-Mock). L-cells expressing nephrin and Neph3 (L-nephrin-Neph3-EGFP) in the same cells showed a minor 1,4-fold increase ($p \leq 0.01$) in aggregation index compared to control cells (L-EGFP). However, L-cells expressing Neph1 or Neph3 alone both showed a significant 2-fold or 2,3-fold increase ($p \leq 0.001$) in aggregation index compared to control cells (L-EGFP and L-Mock), respectively. Furthermore, when L-nephrin cells were used in combination with L-Neph1-HA or L-Neph3-EGFP cells in hanging drop assays, they showed 3-fold increase in aggregation index ($p \leq 0.001$). The co-localization of Neph3-EGFP and nephrin in the cell-cell contacts was confirmed by immunofluorescence staining of the cell aggregates (Study IV, Figure 3). These results suggest that Neph1 and Neph3 are homophilic adhesion molecules and when they trans-interact with nephrin, their adhesion activity is enhanced (Figure 9).

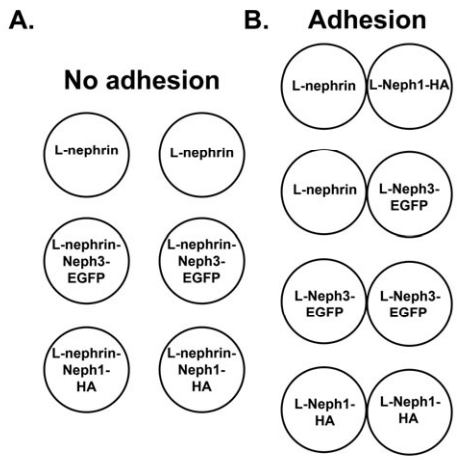

Figure 9: *Summary of the results from adhesion assay. (A) Nephrin alone or when expressed in the same cell with Nehp1 or Neph3 cannot induce cell-adhesion. (B) Trans-interaction between nephrin and Neph1 or Neph3 promotes cell-cell contact formation. Neph1 and Neph3 exhibit homophilic adhesion activity.*

10. *Tyrosine phosphorylation of nephrin is reduced after it forms cell-cell contacts with Neph1 or Neph3*

Since tyrosine phosphorylation of nephrin has been shown to be increased during podocyte development and in injured podocytes lacking proper SD stuctures (Li *et al.,* 2004; Verma *et al.,* 2006; Garg *et al.,* 2007), we investigated whether tyrosine phosphorylation status of nephrin is altered after nephrin forms cell-cell contacts with Neph1 or Neph3. To this end we immunoprecipitated nephrin from hanging drop cultures containing L-nephrin, L-nephrin-Neph1-HA or L-nephrin-Neph3-EGFP cells. In addition, nephrin was immunoprecipitated from hanging drops containing mixed cell populations of L-nephrin and L-Neph1-HA or L-nephrin and L-Neph3-EGFP. To determine nephrin tyrosine phosphorylation, quantitative immunoblot assay was performed by using anti-nephrin and anti-phosphotyrosine antibodies from nephrin immunoprecipitates. The results showed that nephrin tyrosine phosporylation status stayed similar in hanging drops containing L-nephrin, L-nephrin-Neph1-HA or L-nephrin-Neph3-EGFP cells. However, tyrosine phosphorylation of nephrin was reduced by 50 % in hanging drops containing mixed cell populations of L-nephrin and L-Neph3-EGFP or L-nephrin and L-Neph1-HA compared to hanging drops containing L-nephrin cells alone (Study IV, Figure 4). The results indicate that the trans-interaction of nephrin with Neph1 or Neph3, which induces cell adhesion, results in de-phosphorylation of nephrin.

11. Neph3 is up-regulated in nephrin deficient mouse kidneys

Even though the SD cannot form without nephrin, podocytes still make narrow tight-junction like junctions (Putaala *et al.,* 2001; Rantanen *et al.,* 2002). Since we found that Neph1 and Neph3 can induce cell-cell contact formation when expressed in L-cells (Study IV), we investigated the localization and expression of Neph1 and Neph3 in nephrin deficient mouse kidneys. Since podocin expression has previously been shown to be preserved in nephrin deficient mouse kidney, we used it as a control. Immunofluoresence staining showed that Neph3 expression was increased in nephrin deficient mouse kidneys compared to wild-type mice, whereas podocin expression stayed at similar levels as excepted. Quantitative immunoblotting analysis showed 4-fold ($p \leq 0.01$) increase in Neph3 expression in nephrin deficient mouse kidneys compared to controls. Neph1 showed also 2–fold up-regulation, but it didn't reach statistical significance ($p \leq 0.14$), and also podocin protein levels were similar between the genotypes (Study IV, Figure 5). These data suggest that Neph3 may play a role in the formation of tight-junction like structures between nephrin deficient podocytes.

DISCUSSION

Glomerular disorders are a major cause of chronic kidney disease sharing the common features of proteinuria and podocyte injury. The typical characteristics of podocyte injury include disruption of the SD, formation of tight-junction-like structures between the podocyte foot processes and effacement of foot processes. Lack of nephrin in man and mouse results in these typical morphological changes already at birth associated with heavy proteinuria which leads to death without kidney transplantation. This highlights the importance of nephrin and consequently SD in regulating the kidney filtration function. However, the precise molecular mechanism of how nephrin is involved in the formation of the SD is unknown. It is, therefore, of the utmost importance to search for novel nephrin binding proteins and investigate their functional relevance for the formation of the SD.

1. Densin is a novel component of the SD

Densin is a post-synaptic density (PSD) protein of excitatory synapses and it belongs to the LAP (leucine-rich repeat and PDZ domain) protein family containing multiple protein interaction domains including 16 leucine rich repeats, an Arg-Gly-Asp (RGD) tripeptide and a single PDZ-domain (Apperson *et al.,* 1996). Densin is highly concentrated in the PSD and associates with N-methyl-D-aspartate (NMDA) receptor complexes and adherens junction proteins (Strack *et al.,* 2000; Ohtakara *et al.,* 2002; Izawa *et al.,* 2002). In Study I we localized densin to the SD and showed that it interacts with nephrin. The finding that densin can be found both in the SD and synaptic junction makes it an interesting binding partner for nephrin for several reasons. Even though synaptic junction is an asymmetric junction containing presynaptic and post-synaptic sites acting in neurotransmission and the SD is a symmetrical junction forming a molecular sieve, they share some similarities. The synaptic cleft is about 20-50 nm wide (Bear *et al*., 1996) resembling the about 40 nm podocyte slit. In addition, neurons and podocytes are both terminally differentiated cells possessing processes and highly organized actin cytoskeleton (Kobayashi *et al.,* 2004). Most interestingly, in the PSD densin binds to molecules which are found in the nephrin protein complex in the SD including cadherin/catenins (Lehtonen *et al.,* 2004; Izawa *et al.,* 2002) and alpha-actinin-4 (Walikonis *et al.,* 2001; Lehtonen *et al.,* 2004). Densin also interacts with MAGUIN-1 (Ohtakara *et al* 2002), which binds to nephrin interacting protein MAGI-2 (Yao *et al.,* 1999; Lehtonen *et al.,* 2005). Furthermore, in the PSD, MAGI-2 binds to dendrin (Kremerskothen *et al.,* 2006) which interacts with nephrin in the SD (Asanuma *et al.,* 2007). According to these morphological and molecular similarities, densin may share functional properties in the PSD and the SD.

Even though densin was found over ten years ago, still little is known about its function. Densin contains many protein-protein interaction domains and it binds to calcium/calmodulin-dependent protein kinase II (CAmKII) (Strack *et al.,* 2000), a synaptic scaffold protein Shank (Quitsch *et al.,* 2005) and also associates with PSD-95 via MAGUIN-1 (Ohtakara *et al.,* 2002). Therefore, it has been suggested to act as a

scaffold protein organizing NMDA receptor complexes and signalling pathways. Since the nephrin protein complex contains multiple proteins and has been shown to induce signalling, it is tempting to speculate that densin would have a role in organizing the nephrin complexes. In the PSD densin associates also with synaptic adherens junction protein N-cadherin via δ-catenin (Izawa *et al.,* 2002) which in turn suggest that it may play a role in neuronal adhesion. Similarly, in podocytes we showed that densin associates with P-cadherin via β-catenin (Study II). We further showed that densin is found at the plasma membrane only at cell-cell contacts where it co-localizes with β-catenin and F-actin in cultured mouse podocytes (Study II) suggesting that densin may play a role in podocyte cell adhesion. Nephrin binds also to P-cadherin (Lehtonen *et al.,* 2004) indicating that densin and nephrin may be in the same complex with cadherin/catenins. Additionally, nephrin and densin share common connections to the actin cytoskeleton including cadherins/catenins and alpha-actinin-4 (Walikonis *et al.,* 2001; Lehtonen *et al.,* 2004). The interaction with alpha-actinin-4 is particularly interesting, since it has a crucial role in organizing podocyte actin cytoskeleton (Kaplan *et al.,* 2000; Weins *et al.,* 2005) (Figure 10). In cultured hippocampal neurons densin has been shown to induce branching (Quitsch *et al* 2005) indicating that densin may balance neurite elongation and branching. This kind of function for densin was not found in podocytes, since over-expression of densin in cultured mouse podocytes did not induce formation of cellular processes (Study II).

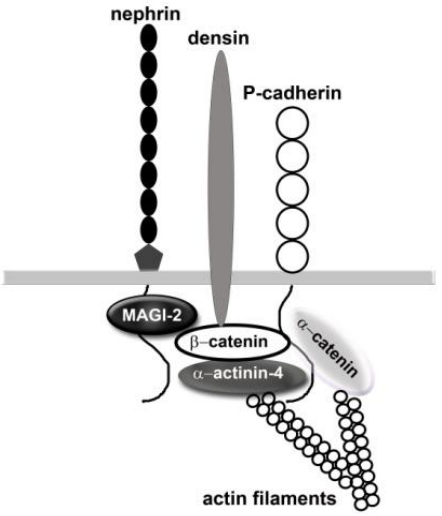

Figure 10. *Schematic presentation of the proposed densin-nephrin complex and its connections to podocyte actin cytoskeleton.*

2. Does densin regulate the formation of adherens junctions?

Other members of LAP protein family, Scribble and Erbin, have been shown to regulate the formation of the adherens junctions. Scribble is transported to MDCK cell junctions in E-cadherin dependent manner (Navarro *et al.,* 2005) and it facilitates the formation of adherens junctions in MDCK cells by stabilizing the connection between cadherins and catenins, and consequently improving their connections to actin cytoskeleton (Qin *et al.,* 2005). Erbin also regulates the interaction between β-catenin and E-cadherin via mitogen-activated protein kinase in Schwann cell contacts (Rangwala *et al.,* 2005). We showed that densin localization at the plasma membrane was dependent of cell-cell contacts as well as β-catenin and F-actin (Study II). Furthermore, densin and β-catenin were similarly translocated to intracellular vesicles during junction disruption. Upon cell-cell contact formation, densin and β-catenin were first found in early junctions in which cadherins/catenins are clustered into puncta and later they localized in mature continuous belt-like junctions (Study II) (Adams *et al.,* 1998). These results further confirm that densin binds to adherens junction proteins and also indicate that it may play a similar role as other LAP members in regulating adherens junction assembly. Interestingly, densin has been shown to localize to adherens junctions between sertoli cells and developing germ cells in the testis (Lassila *et al.,* 2007). However, the molecular mechanisms of how densin participates in the formation of adherens junctions requires further investigation.

Interestingly, LET-413, the *C.elegans* orthologue of densin, plays a role in the formation and proper positioning of adherens junctions which is important for polarization of the epithelium of the *C.elegans* embryos (Legouis *et al.,* 2000). Podocytes are highly polarized cells and the lateral position of the SD is important for separating positively charged apical site from the basal site attached to the GBM.Thus, densin may contribute to the polarization of podocyte foot processes and correct positioning of the SD by regulating the proper assembly of cadherin-nephrin protein complexes as well as their connections to actin cytoskeleton.

Several conditionally immortalized podocyte cell lines have been established to investigate the role of podocyte proteins. Mouse podocyte cell lines have been produced from isolated glomeruli of mice, harbouring the temperature-sensitive SV40 large T antigen (Schiwek et al., 2004; Mundel *et al.*, 1997). A human podocyte cell line has been developed from isolated glomeruli by transfection with the temperature-sensitive SV40 large T antigen (Saleem *et al.,* 2002). This allows podocytes to proliferate at 33 ºC and differentiate at 37 ºC. Even though, in the differentiated stage, the cultured podocyte cells show formation of cellular processes and cell-cell contacts (Schiwek et al., 2004; Mundel *et al.*, 1997; Saleem *et al.,* 2002), morphologically they do not superimpose podocytes *in vivo.* Furthermore, in the glomerular filtration barrier podocytes function in cooperation with the GBM and glomerular endothelial cells. Therefore, one should be cautious in interpreting results obtained with cultured podocytes to *in vivo* podocytes. This should also be taken into consideration in our

results which suggest that densin and adherens junction proteins may regulate the SD as our investigations were performed on cultured mouse podocytes.

3. *β-catenin is not essential for formation or maintenance of the SD*

Cadherin-dependent cell adhesion is initiated when cadherins on opposite cell surfaces interact. This induces cadherin clustering into puncta and later maturation into belt-like junctions with strong adhesion. The strength of the cadherin adhesion is dependent on the connection of the adhesion complex to actin cytoskeleton. β-catenin provides a link for cadherins to cytoskeleton by binding to actin binding and organizing protein alpha-catenin (Aberle *et al.,* 1994; Nelson, 2008). SD contains two classical cadherins, P- and VE-cadherins, which are connected to actin cytoskeleton via catenins (Reiser *et al.,* 2000; Cohen *et al.,* 2006). Furthermore, densin and nephrin associate with the P-cadherin/catenin complexes in the SD (Study II; Lehtonen *et al.,* 2004). The important role of β-catenin in linking cadherins to cytoskeleton led us to hypothesize that β-catenin may have an important role in maintaining the SD. However, deletion of β-catenin from adult mouse podocyte did not cause disruption of the SD which showed that β-catenin in not essential for maintaining the SD structure (Study III). Recently, it has been shown that deletion of β-catenin in capillary loop stage of podocyte differentiation did not cause any morphological alterations in podocytes (Dai *et al.,* 2009) which shows that β-catenin is neither needed for the formation of the SD. Whether β-catenin plays a role in the formation of epithelial junctions during S-shaped body stage of glomerular differentiation remains still unknown. The lack of β-catenin may be compensated by structurally homologous protein, γ-catenin, which in other cell types has been shown to take over the function of β-catenin in cadherin-dependent cell adhesion (Huelsken *et al.,* 2000).

4. *β-catenin plays a role in the modulation of SD in podocyte injury*

β-catenin and cadherins are expressed at a higher level during podocyte differentiation in S-shaped body and capillary loop stages than in mature podocytes (Yaoita *et al.,* 2002; Usui *et al.,* 2003; Ruotsalainen *et al* 2000). Some authors have been unable to detect β-catenin in mature podocytes (Yaoita *et al.,* 2002; Usui *et al.,* 2003), whereas others have reported it to be present in mature podocytes (Piepenhagen and Nelson, 1995). This discrepancy is most likely due to differencies in the sensitivities of the antibodies used in the studies. However, it also indicates that β-catenin is expressed at low levels in the mature podocytes as we also showed in Study III. Since injured podocytes mimic immature podocytes in having broad foot processes which are connected by tight-junction like structures and apically located SDs, we wanted to test whether β-catenin would play a role in the transformation of the SD into these junctions during podocyte injury. We showed that in a way similar during podocyte

development, β-catenin expression was up-regulated in podocytes in adriamycin nephropathy which is characterized by foot process effacement, formation of tight-junction like structures and apically dislocated SDs (Study III). Most interestingly, in β-catenin deficient mice the level of foot process effacement and albuminuria were lower than in control mice. These results are further supported by the recent data showing similar findings in β-catenin deficient mice after adriamycin treatment (Dai *et al.,* 2009) suggesting that β-catenin plays a role in disruption of the SD in adriamycin nephropathy.

Similarly as β-catenin, the cadherin superfamily member Fat1 is also down-regulated during podocyte maturation and up-regulated in injured podocytes in PAN model. Furthermore, it co-localizes with cadherin/catenin complex during podocyte differentiation (Yaoita *et al* 2005) and is able to interact with β-catenin (Hou *et al.,* 2006). β-catenin interaction partner densin is up-regulated in podocytes of CNF patients showing foot process effacement and formation of tight-junction like structures (Study I). Therefore, β-catenin, densin and Fat1 may participate in the same molecular pathways which are essential for the formation of the modified junctions in injured podocytes. This should be, however, investigated in detail in each disease model, since the podocyte injury is induced differently in each of them.

Nephrin has been shown to be down-regulated in various acquired human kidney diseases (Wang *et al.,* 2002; Doublier *et al.,* 2001; Koop *et al.,* 2003) as well as in various experimental animal models (Luimula *et al.,* 2000; Yuan *et al.,* 2002). Nephrin down-regulation has been further shown to correlate with foot process effacement in human acquired kidney diseases (Koop *et al.,* 2003; Huh *et al.,* 2002). These data indicate that nephrin down-regulation can be used as a marker for podocyte injury. Our study III showed that β-catenin deficient and control mice showed similar expression of nephrin after adriamycin treatment even though the degree of foot process effacement was higher in control mice. However, it has also been demonstrated that nephrin expression is reduced in glomeruli of control mice compared to β-catenin deficient mice after adriamycin treatment (Dai *et al.,* 2009). In this study a higher dose of adriamycin was used than in our study, which resulted in more severe albuminuria and podocyte injury which may explain the down-regulation of nephrin in the another study. In our study foot process effacement was not homogenous, which is also a common finding in human glomerular diseases. This may also explain why alteration in the expression of nephrin in several acquired kidney diseases have not been detected when the overall expression level instead of individual podocyte expression level has been investigated (Patrakka *et al.,* 2001). Ours and other studies may therefore indicate that the down-regulation of nephrin expression is not a sensitive marker for foot process effacement but rather reflects an advanced stage of podocyte foot process injury.

In addition to serving as a link between cadherins and cytoskeleton, β-catenin also plays a role in the regulation of transcription in Wnt-signalling cascade. In this cascade, secreted Wnt glycoproteins bind to Frizzled receptor proteins which leads to stabilization of β-catenin by preventing its phosphorylation and degradation. Consequently, the level of cytosolic β-catenin rises and β-catenin translocates into

nucleus in which it binds to lymphoid enhancer factor/T cell factor (LEF/TCF). This complex turns on the transcription of Wnt-response genes which have been shown to play a role in diseases and self-renewal of various tissues (Clevers, 2006) as well as in the development of various organs including kidney (Merkel *et al.,* 2007). In the mature podocyte the Wnt signalling is silenced (Iglesias *et al.,* 2007), whereas after adriamycin treatment β-catenin is reported to be translocated to podocyte nucleus indicating activation of Wnt signalling (Teixeira *et al.,* 2005; Dai *et al.,* 2009). We could not detect β-catenin in the podocyte nucleus or up-regulation of Wnt signalling markers dephosphorylated β-catenin or phosphorylated GSK-3β (Study III). In other studies BALB/c mice have been used, which are more sensitive to adriamycin and develop more severe podocyte injury and albuminuria than C57BL/6J strain in our study (Teixeira *et al* 2005). In addition, a higher dose of adriamycin has been used in C57BL/6J strain than we used, which also resulted in higher albuminuria (Dai *et al.,* 2009). Therefore, it may be that more severe podocyte injury is needed to induce Wnt-signalling cascades. However, our study may indicate that Wnt-signalling is not an early marker for podocyte injury in adriamycin nephropathy.

Even though the studies on podocyte specific β-catenin deficient mice by us and others clearly demonstrated that podocytes play a central role in the development of albuminuria in adriamycin nephropathy (Study III; Dai *et al.,* 2009) it has, recently, been shown that endothelial cell are also affected in this model. Adriamycin was shown to cause thinning of the endothelial cell glycocalyx by down-regulating certain proteoglycans and glycosaminoglycans. This was accompanied by reduction of charge selectivity of the glomerular filter which was suggested to contribute to the development of albuminuria in adriamycin nephropathy (Jeansson *et al.,* 2009). These results suggest that podocytes may not be the only target for adriamycin cytotoxicity. Furthermore, it is worth to mention that endothelial and mesangial cells express also β-catenin and therefore Wnt-signalling may be activated in these cells also upon adriamycin induced injury which has not been investigated in detail.

Adriamycin is an anthracycline antibiotic, which has been used for the treatment of solid tumors due to its cytostatic and cytotoxic actions on cells. These actions have been shown to be mediated by the formation of free radicals, induction of apoptosis, lipid peroxidation and induction of DNA damage (Gewirtz, 1999). However, the use of adriamycin in chemotherapy has been decreased due to its diverse toxic effects including cardiac and renal toxicity (Yilmaz *et al.,* 2006). Adriamycin treatment results in podocyte injury in the mouse which shares characteristics of podocyte injury detected in human kidney diseases including podocyte effacement and narrowing of the slits between podocytes. However, since adriamycin is an antitumour antibiotics, the mechanism how it induces podocyte injury is apparently not be same that induces podocyte injury in most human kidney diseases. This should be kept in mind when considering our results which show that β-catenin plays a role in the development of podocyte injury and albuminuria after adriamycin treatment (Study III).

5. *Nephrin needs to trans-interact with Neph3 or Neph1 in order to induce cell adhesion*

Neph3 shares homology with nephrin, localizes to the SD and, similarly as nephrin, is down-regulated in human acquired kidney diseases (Ihalmo *et al.,* 2003; Ihalmo *et al.,* 2007) indicating that Neph3 may interact with nephrin. We showed in Study IV that similarly as Neph1-2 (Liu *et al.,* 2003; Gerke *et al.,* 2003; Gerke *et al.,* 2005; Barletta *et al.,* 2003), Neph3 forms homodimers and heterodimers with nephrin. In addition, we showed that Neph3 was able to bind to Neph1. These results indicate that Neph3 may together with nephrin and Neph1 participate in the formation of the SD.

The *C.elegans* and Drosophila orthologues of nephrin and Neph family members have been shown to induce cell adhesion by forming homodimers or heterodimers between opposite cell surfaces (Dworak *et al.,* 2001; Bour *et al.,* 2000; Ruiz-Gomez *et al.,* 2000; Strunkelnberg *et al.,* 2001; Dworak, 2002; Bao *et al.,* 2005; Carthew *et al.,* 2007; Shen and Bargmann, 2003; Shen *et al.,* 2004). We demonstrated that both mouse Neph1 and Neph3 showed homophilic adhesion activity, whereas nephrin was not able to induce cell adhesion alone. However, nephrin was able to form cell-cell contacts after trans-interacting with Neph1 or Neph3. Interestingly, these trans-interactions induced higher adhesion activity than Neph1 or Neph3 were able to induce alone. In addition, cell adhesion was induced only when nephrin bound to Neph1 or Neph3 on the opposite cell surfaces but not when nephrin was present together with Neph1 or Neph3 in the same cell. Furthermore, the expression of nephrin in the same cell with Neph1 or Neph3 inhibited their adhesion activity. This may indicate that the heterophilic cis-interaction of Neph1 or Neph3 with nephrin may block the extracellular binding domain which mediates the binding of these proteins to each other on the opposite cell surface. Since nephrin, Neph1 and Neph3 are all expressed in the SD, our finding, that simultaneous expression of nephrin with Neph1 or Neph3 in the same cell inhibits their adhesion activity, might be considered paradoxical. However, one should keep in mind that these adhesion assays were accomplished in a simple cell culture model using L-cells, which lack most SD components. Since the SD is considered to be a dynamic junction, there may exist signalling pathways in podocytes which regulate whether nephrin interacts in cis or in trans configuration with Neph1 or Neph3. These pathways could be important for regulating podocyte intercellular adhesion and SD connections to actin cytoskeleton which may be important for maintaining the SD.

It has been shown that nephrin expression in HEK293 cells induces formation of cell clusters and therefore it has been suggested that nephrin is a homophilic adhesion molecule (Khoshnoodi *et al.,* 2003). Recently, it has also been shown by using Jurkat cells that nephrin is unable to induce cell adhesion in trans configuration with Neph3 (Nishida *et al*., 2010). These results are in contrast to our findings which showed that nephrin was able to induce cell adhesion only after binding in trans configuration to Neph1 or Neph3 (Study IV). The discrepancy between the results may be explained by the different cells used in the studies. HEK293 cells express adherens junction proteins and are, therefore, able to form cell-cell contacts (Khoshnoodi *et al.,* 2003). Jurkat cells,

in turn, are T-lymphocytes (Nishid *et al*., 2010). We chose mouse L-fibroblasts for our study since they lack expression of adherens junction proteins and thus cannot form cell-cell contacts. Furthermore, L-cells have successfully been used to investigate adhesion activity of other Ig superfamily proteins including nectins (Satoh-Horikawa *et al.,* 2000) further supporting that L-cells are a relevant model to investigate adhesion activity of nephrin, Neph1 and Neph3.

6. *Tyrosine phosphorylation of nephrin is reduced after nephrin forms cell-cell contacts with Neph1 or Neph3*

Nephrin is a signalling molecule which is able to induce actin polymerization (Zhu *et al.,* 2008; Verma *et al*., 2006; Jones *et al.,* 2006; Li *et al*., 2001), elevate cytosolic calcium levels (Harita *et al*., 2009), induce raft-mediated endocytosis (Qin *et al*., 2009) and decrease apoptosis (Huber *et al.,* 2003). The signalling is initiated by clustering of nephrin which leads to tyrosine phosphorylation of nephrin by Fyn. Tyrosine phosphorylation of nephrin has been shown to be increased during podocyte development as well as in experimental animal models including Heymann nephritis, protamine sulphate nephrosis and PAN (Li *et al.,* 2004; Verma *et al.,* 2006; Garg *et al.,* 2007). During the formation of podocyte foot processes and in podocyte injury, podocytes undergo major morphological alterations which associate among other cellular processes with rearrangement of podocyte actin cytoskeleton. Therefore, it has been suggested that once SD has been assembled, in steady state situation less nephrin signalling is needed to induce for instance actin polymerization. Our results support this hypothesis by showing that tyrosine phosphorylation of nephrin is decreased after it forms cell-cell contacts with Neph1 or Neph3. However, it has also been shown that nephrin is tyrosine phosphorylated in mature podocytes (Jones *et al*., 2009). In addition, it has been shown that tyrosine phosphorylation of nephrin is decreased in MCD and in PAN model (Uchida *et al.,* 2008) suggesting that the tyrosine phosphorylation of nephrin is important for the maintenance of the SD. Taken together, since SD is considered as a dynamic cell junction, it may be that the balance between tyrosine phosphorylation and de-phosphorylation of nephrin may be critical for maintaining the integrity of the SD and consequently podocyte morphology.

7. *Lack of nephrin leads to up-regulation of Neph3*

Without nephrin the SD cannot be formed and podocyte foot process slits are narrowed. We found that Neph3 was up-regulated in nephrin deficient mouse kidneys. Also Neph1 showed tendency toward up-regulation, which didn't, however, reach statistical significance (Study IV). Since we showed that Neph1 and Neph3 are homophilic adhesion molecules, it may indicate that without nephrin they would participate in the formation of narrow junctions. Actually, the extracellular domains of Neph1 and Neph3 are shorter than that of nephrin supporting that they could form more narrow junctions alone than together with nephrin (Sellin *et al.,* 2003; Ihalmo *et al.,* 2003). Furthermore, the adhesion activity of nephrin-Neph1 or nephrin-Neph3 trans-interactions was higher than Nehp1 or Neph3 could induce alone (Study IV)

suggesting that different molecular adhesion machineries could be activated after the interactions. Therefore, these findings may explain why nephrin-like proteins Neph1 or Neph3 cannot compensate for the loss of nephrin or vice versa. The up-regulation of Neph3 in nephrin deficient mouse kidneys also suggests that the regulation of the expression of Neph3 and presumably also Neph1 is important for maintaining the proper SD structure.

Interestingly, Neph3 is up-regulated in the postmitotic neural precursor cells in the developing spinal cord and down-regulated during maturation of neurons (Minaki *et al.,* 2005). Similarly, Neph3 has been shown to be up-regulated in developing podocytes and down-regulated during podocyte maturation in zebrafish (unpublished data). We did not compare the expression of Neph3 between immature and mature podocytes in mouse, but based on the finding that it is up-regulated in nephrin deficient mouse podocytes which mimic podocytes during development, it is possible that such down-regulation may also exist in mouse and man. Cadherin/catenin complex is also down-regulated during podocyte maturation (Yaoita *et al.,* 2002; Usui *et al.,* 2003; Ruotsalainen *et al.,* 2000). Interestingly, Neph3 has been co-localized with adherens junction components including ZO-1 and N-cadherin in adherens junctions of early postmitotic precursor cells in developing spinal cord (Minaki *et al.,* 2005). Furthermore, nephrin has been shown to bind to adherens junction components (Lehtonen *et al.,* 2004). It would, therefore, be interesting to investigate whether Neph3 exists in the same complex with adherens junction proteins during podocyte differentiation or in mature podocytes.

CONCLUSIONS

Nephrin is a crucial structural and signalling protein of the SD and consequently critical for maintaining the permselectivity of the glomerular filtration barrier. Therefore, it is important to understand the molecular mechanisms of how nephrin participates in the formation of the SD. In addition, it is important to identify the molecular pathways which are involved in the down-regulation of nephrin and the disruption of the SD. This thesis work has searched for novel nephrin interaction partners and identified densin and Neph3 as components of the nephrin protein complex. The role of densin and Neph3 were further investigated by using various cell culture and mouse models.

In this thesis work the LAP protein family member densin was localized to the SD and further shown to form a complex with nephrin and adherens junction proteins β-catenin and P-cadherin. Densin was also shown to behave in a similar fashion as adherens junction proteins in cell-cell contacts suggesting that it may function in cell adhesion similarly as other members of the LAP family. However, whether densin is needed for the formation of the SD remains unknown and to clarify this, a podocyte specific densin knock-out mouse should be established. Deletion of β-catenin, an interaction partner of densin, specifically in podocytes of adult mice resulted in normal podocyte phenotype. This shows that neither β-catenin nor the interaction between densin and β-catenin are necessary to maintain the SD.

This thesis work also showed that the SD protein Neph3 homodimerizes and binds to nephrin and Neph1. The results indicate that Neph3 has similar binding properties as the other members of the Neph family, Neph1-2, and therefore suggest that Neph1-3 and nephrin may function together in the SD. The most interesting result in this thesis work was that Neph1 and Neph3 were able to induce cell adhesion alone, whereas nephrin needed to interact either with Neph1 or Neph3 in trans-configuration in order to establish cell-cell contacts. Furthermore, the heterophilic trans-interactions induced de-phosphorylation of nephrin. The molecular pathways which are activated upon these interactions require further investigation. In addition, it is important to identify the specific kinases and phosphatases and associated signalling cascades which regulate tyrosine phosphorylation of nephrin during cell-cell contact formation. Finally, in order to know whether Neph3 is crucial for the formation of the SD, a podocyte specific Neph3 knock-out mouse should be generated.

To investigate podocyte injury in kidney tissue from CNF patients, a nephrin deficient mouse line and adriamycin nephropathy model in mouse were used. All these models share common characteristics of podocyte injury including foot process effacement and replacement of SD with tight junction-like structures. The main difference between these models is that loss of nephrin in man and mouse leads to a phenotype in which SDs cannot be formed, whereas in adriamycin nephropathy model the mature SDs are disrupted. This study showed that densin and Neph3 were both up-regulated in nephrin deficient podocytes. β-catenin, in turn, was up-regulated in adriamycin treated

podocytes. Further, by using a podocyte-specific β-catenin knock out mouse we found that β-catenin contributes to the formation podocyte injury in adriamycin nephropathy.

The finding that densin, Neph3 and β-catenin are all part of the nephrin protein complex and up-regulated when podocytes are effaced and fused, makes it tempting to speculate that they would participate in common molecular mechanisms which lead to these morphological changes. Particularly interesting would be to know whether β-catenin-densin or Neph3-nephrin interactions play a role in the molecular pathways leading to podocyte injury. To this end these interactions should be analyzed in experimental animal models. Finally, up-regulation of densin, β-catenin and Neph3 in injured podocytes indicates that regulation of their expression is important to sustain proper SD structure and therefore emphasizes the importance to investigate their transcriptional regulation.

Nephrin has been previously shown to bind to the Ig superfamily and adherens junction proteins and these interactions have been suggested to play a role in SD assembly. This thesis extended the current knowledge of the molecular architecture of the nephrin protein complex by showing that Ig superfamily member Neph3 and adherens junction protein-associating protein densin are in complex with nephrin. Furthermore, the data presented offer a novel insight into the role of nephrin, Neph1 and Neph3 in cell adhesion which may play a role also in the formation of the SD. Finally, the alterations in the expression of Neph3, densin and β-catenin in injured podocytes may give novel insights into molecular mechanisms involved in the development proteinuria.

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